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Research Laboratories, Parke, Davis & Company, Detroit, Michigan

ELECTRON MICROSCOPY OF POLIOMYELITIS VIRUS

The infectious capacity of poliomyelitis virus has been shown to be associated with spherical particles approximately 30 mμ in diameter.[7, 10, 12, 13] The present study suggests that these particles, like those of other animal viruses recently studied[1, 2, 9] consist of an outer membranous structure surrounding an internal nucleus-like body.

The ultimate size and shape of small virus particles as they are seen in shadowed electron micrographs appear to be dependent upon the hydration of the particles at the time of the first exposure to high vacuum. By the use of a collodion-over-agar filtration technique it has been possible to prepare and mount virus suspensions directly from saline media and obtain microscope specimens which are free of salt crystals and osmotic distortion. Such preparations preserve the three-dimensional structure of the cell-like particles. Through variations in the drying time, the saline media, and through inclusion of hygroscopic substances in the media, the degree of hydration prior to the initial vacuum drying can be altered. Representative strains of poliomyelitis virus (Mahoney, Type 1; MEF-1, Type 2; and Saukett, Type 3) have been studied; comparisons of shadowed and unshadowed electron micrographs have revealed internal detail and morphology within poliomyelitis virus particles.

METHOD

Concentrated poliomyelitis virus preparations containing essentially one type of particle have been obtained consistently from infectious tissue culture fluids by means of a combination of alcohol precipitation, Sharples and ultracentrifugation. Details of the purification methods and the characterization of the fully active concentrates will be the subject of a forthcoming communication. The collodion-over-agar technique for the preparation of electron microscope specimens is described below; it is based upon the work of Kellenberger[4] and the pseudo-replica and agar sedimentation techniques developed by Sharp.[10, 11]

Two per cent agar (Difco-Bacto) in distilled water heated to boiling is filtered through Pyrex wool into four-inch petri dishes. The agar suspension, three to four

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† Assistant Research Microbiologist.
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mm. thick, is allowed to solidify in the open dishes and remain undisturbed six inches below an ultraviolet lamp (Westinghouse G15T8) for 12 to 18 hours (usually overnight); during this interval the agar surface hardens considerably and the whole mass shrinks somewhat. The dishes are then covered and stored at room temperature. They may be used for several days; satisfactory preparations have been made with agar six to seven days after the plates are poured; usually, however, such agar has become too dry to handle well, or mold formation has occurred.

The agar mass is loosened around the rim and the petri dish is then inverted into a larger petri dish where the circular mass of agar readily drops, exposing the original agar-to-glass surface. Approximately 1.5 cm. square blocks of agar are cut and picked up with a Bard-Parker blade or spatula and transferred to individual microscope slides which are tapped gently on the work surface to seat the agar block. The slide with the agar block is then placed in a shallow covered dish covering a small open petri dish of "Drierite," a moisture absorbent, where it is allowed to remain from five to ten minutes; this hardens and surface-dries the freshly exposed agar surface. The agar block is then flooded with a drop or two of collodion diluted one to ten in freshly distilled amylacetate, and immediately placed in a vertical position to drain. After about 30 seconds the excess of collodion collecting on the lower edge of the agar block is picked off with a torn filter paper edge. After drying for five to ten minutes the slide with the collodion-covered blocks is placed horizontally and a small drop of the preparation to be examined is applied by allowing it to fall on the collodion surface from a fine-tipped pipette. If the collodion film is reasonably intact, the drop will stand up well initially and gradually begin to flatten after a minute or two. The preparation is left undisturbed for three to four minutes and then all but a slight film of moisture wetting the surface is picked off with a torn edge of a piece of filter paper, care being taken not to touch the collodion. The collodion surface dries rapidly, or rather the fluid and salts diffuse into the agar below the collodion, leaving all particulate and nondiffusible material on the collodion surface.

As soon as the last trace of fluid sheen disappears from the collodion surface, the position and area occupied by the original drop of fluid is still readily distinguishable by the slight elevation of the collodion; this area is watched carefully and as soon as it appears to flatten out and become confluent with the surrounding area, the edges of the agar block are quickly cut vertically on all four sides with a sharp knife, reducing the over-all size of the block to approximately one square centimeter. The area occupied by the sample drop is kept approxiately in the center of the block and the collodion film is quickly floated on a clean water surface. A gentle breath on the floated square of collodion locates the specimen area and this is picked up from below in the center of a loop slightly larger than a specimen screen (11 mm. o.d. x 5 mm. i.d. brass washer soldered to wire handle). After lifting the collodion film from the water surface the loop is held in a vertical position and the fluid wetting the surface is led off carefully by capillarity into a torn edge of filter paper; the collodion itself must not be touched. The film is then lowered, specimen side up, upon a specimen screen previously placed upon the upper end of a stainless steel (s.s.) peg. The film should be lowered as nearly parallel to the screen as possible. It should be emphasized that the contact of the square of film with water after the initial drying is only a matter of seconds, with the virus particles on the upper surface of the film away from actual contact with the water.

The upper end of the $\frac{3}{32}$-inch s.s. pegs $\frac{5}{8}$ inches long used to support the screens are turned down to a $\frac{1}{8}$-inch diameter for approximately $\frac{1}{8}$ inches. The subsequent transfer of the collodion-coated specimen screens to specimen caps is accomplished by placing the cap over the screen and peg, inverting, and lifting the peg out. The pegs are mounted in rows of three to six in snugly fitting holes drilled in short pieces of $\frac{1}{2}$-inch square bar stock.

The preparations may be metal-shadowed or examined directly; shadowing is done in this laboratory with the screens on their respective pegs. In fact, for optimal results in retaining the three-dimensional structure of the particles no more than three specimens are made at a time, and the pegs carrying the screens in the bar stock mounts are transferred as soon as possible after being picked up from the water surface to the vacuum chamber of the shadowing equipment.

RESULTS

Figure 1 shows a representative electron micrograph of purified poliomyelitis virus, Type 1 Mahoney strain, prepared as described. It is evident from the shape of the shadows that the particles are essentially spherical. The average particle diameter from the measurement of several hundred individual particles is 29.2 mμ based on direct internal calibration with polystyrene latex. The quotient of shadow length divided by particle diameter for polystyrene latex particles is 4.9; the corresponding ratio for virus particles on the same negative is 4.8. The virus preparation used to prepare the specimen screen of Figure 1 is suspended in Ringer solution, but the type of salts or the concentration present in the media, up to physiological molarities, is not critical. Equally good microscope preparations of strains belonging to all three poliomyelitis virus types are obtained routinely from normal physiological saline buffered at pH 7.2 with 0.05 M phosphate.

In Figures 2 and 3 are shown unshadowed and shadowed Type 3 Saukett strain virus at the same magnification. The virus apparently contains material especially opaque to electrons. The edges of the unshadowed particle images are not clearly defined. If the shadowed and unshadowed micrographs are compared, it will be noted that the unshadowed images appear to be quite dense and to be approximately one-half to two-thirds the over-all size of the shadowed particles. This electron opaque phenomenon was first noted in particles lying in the shadows of polystyrene latex particles and in the depressions in collodion films where they were not reached by the shadow-casting metal. A tentative explanation of this internal particle density is available in preliminary chemical analyses of purified Type 1 Mahoney virus; the nitrogen/phosphorus ratio of 12.5/3.0 would indicate that poliomyelitis virus could contain as much as 30% nucleic acid, provided that all of the phosphorous is present in this form.

The particles seen in Figure 3 are partially dehydrated before the shadowing process. This is accomplished by allowing prolonged contact of the collodion membrane with the agar after all visible evidence of surface moisture has disappeared. One edge of the agar block is cut and the slide with the preparation replaced in the covered dish containing "Drierite" for 30 minutes. The agar block shrinks appreciably during this interval due to loss of moisture. The particle contours in the micrograph indicate a marked flattening of the particles due to the osmotic withdrawal of water, and there is an indication of the dense internal structure; in fact, an apparently rigid, nucleus-like structure is revealed protruding above the collapsed exterior of the particles. Comparison of Figures 2 and 3 suggests that the dense electron-opaque image seen in Figure 2 is probably associated with the centrally located elevated portion of the dehydrated particles shown in Figure 3.

If the poliomyelitis virus particle is a cell-like structure surrounded by a water permeable membrane, it should be possible to alter the characteristics of the external membrane by the inclusion in the media of hygroscopic materials such as glycerol or triethylene glycol. To test this hypothesis a sample of the virus solution, three to four times more concentrated than that used to prepare the above specimens, was added to three to four volumes of 0.25 per cent glycerol. After it has been standing 36 to 96 hours in the refrigerator, increasing numbers of the small bodies are observed in the microscope specimens. The specimen from which Figure 4 was taken had been in the glycerol medium four days and it will be seen that the majority of the particles are altered, having become smaller in diameter. They range from the nearly complete 30 mμ particles (shown in Figs. 1 and 3) to particles having a size of the central bodies (shown in Fig. 2). These smaller structures seen in Figure 4 probably represent those discernible in Figure 3 protruding above the collapsed membrane-like exterior. These particles derived from the 30 mμ virus bodies are variable in size, but average approximately 20 mμ in diameter, the size of the opaque central bodies of Figure 2.

In Figure 5 is seen another preparation of purified Type 1 Mahoney strain virus prepared for electron microscopy by means of the same technique as was used for the preparation of Figure 3. The magnification is 98,000 times. The concentration of virus particles was made somewhat higher than in Figure 3 so that there would be a greater profusion of virus bodies on the final preparation. In this manner, it was planned that some particles would lie in the shadows cast by others. Consistently, in such preparations a collapsed membrane has been observed. A particle image wholly or partially within the shadow of others is seen to have a raised

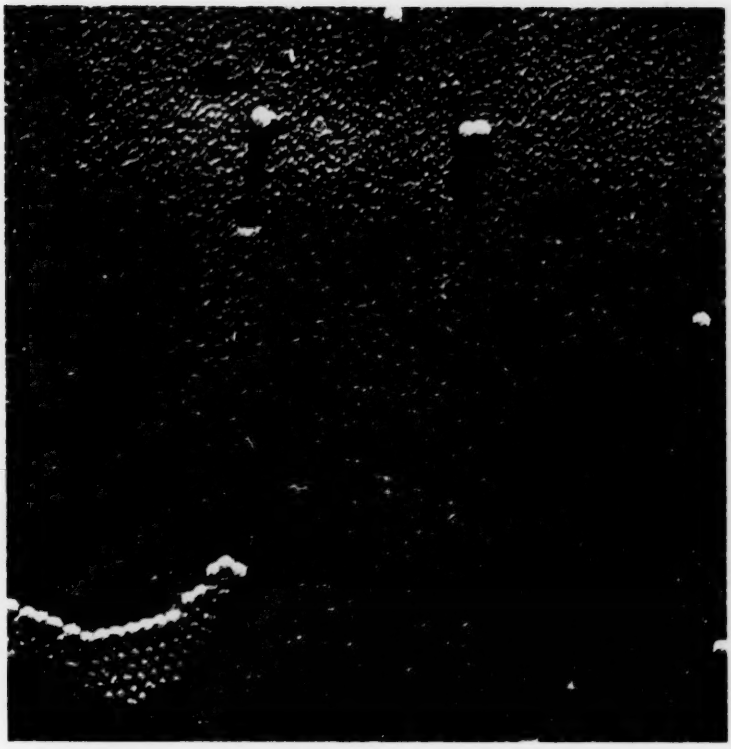

FIG. 1. Poliomyelitis virus, Type 1 (Mahoney), collodion over-agar filtration mount. Specimen transferred to shadower with minimum of osmotic drying; individual particles are uniform in size (30 millimicrons) and nearly spherical. In the closely packed array, particle diameter is about 20% less than that of single particles. ×85000

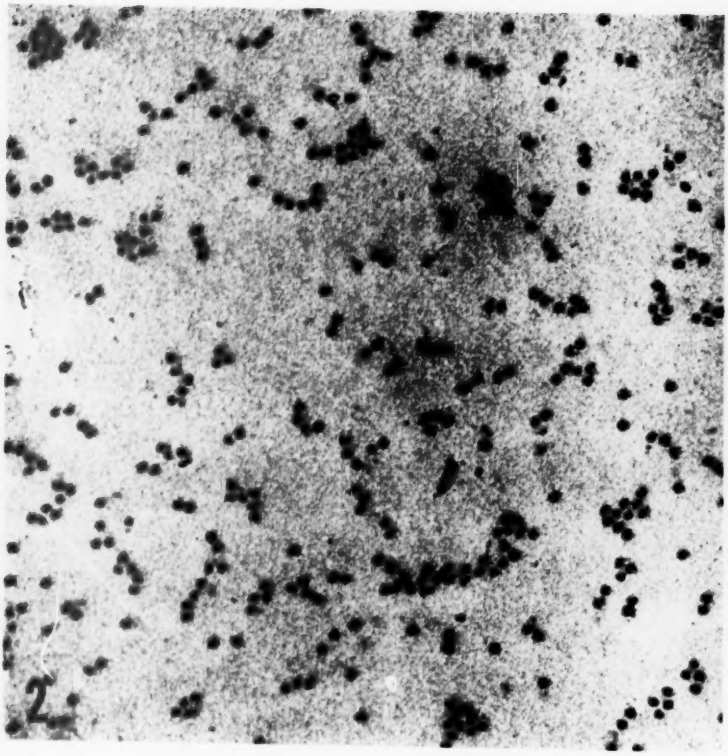

FIG. 2. Poliomyelitis virus, Type 3 (Sankett), unshadowed. The transmission particle image is one-half to two-thirds the size of the shadowed image. Definite spacing is observable in the closely packed arrays, and the dense electron-opaque image suggest an internal nuclear like structure. ×85000

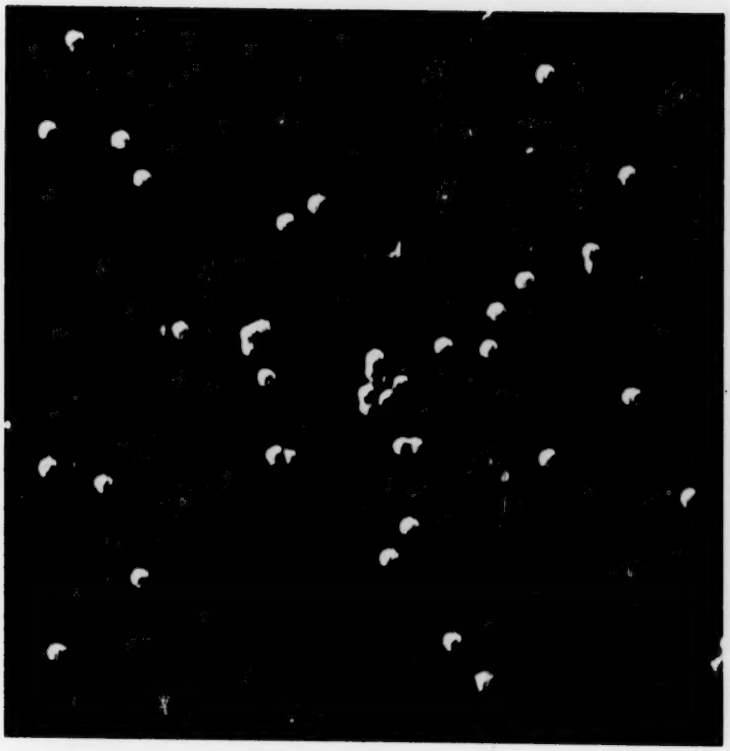

FIG. 3. Poliomyelitis virus, Type 3 (Sanketti), shadowed after dehydration by allowing prolonged contact of the collodion membrane with the agar. Particles are flattened and the dense internal structure is visible protruding above the collapsed membranes. ×85000

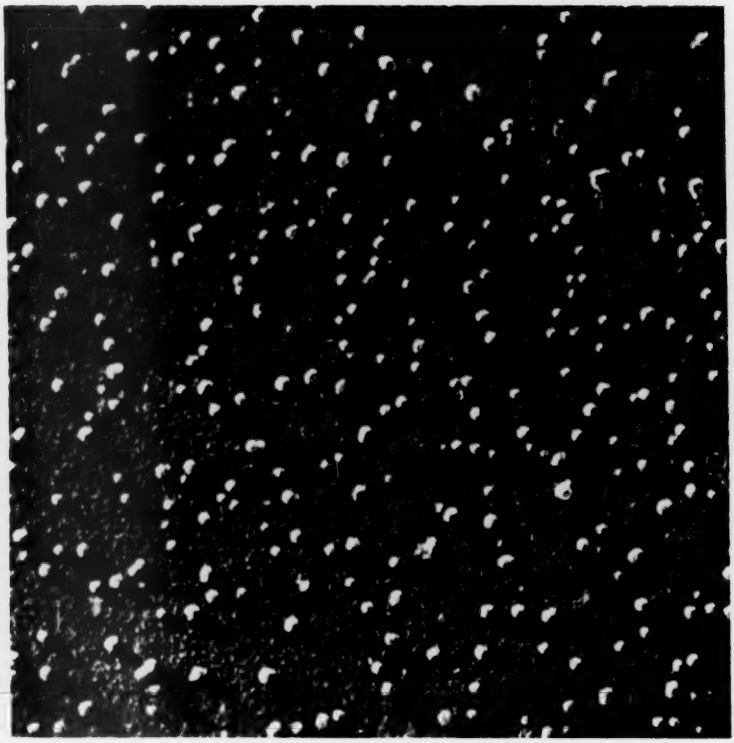

FIG. 4. Poliomyelitis virus, Type 3 (Saukett). This virus preparation was treated with 0.25 per cent glycerine for 96 hours. The virus has undergone changes which are revealed in varying stages of its disintegration. Many particles approximately 20 millimicrons in diameter are visible. x85000

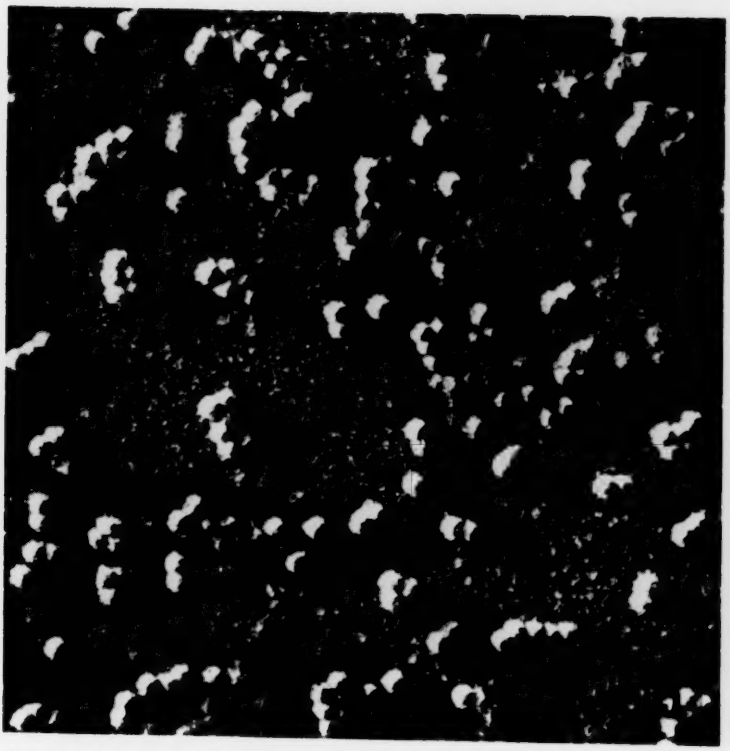

FIG. 5 Poliomyelitis virus, Type 1 (Mahoney), shadowed after dehydration. Particle images in the shadows show the raised central portion surrounded by a flattened membrane. 980000×

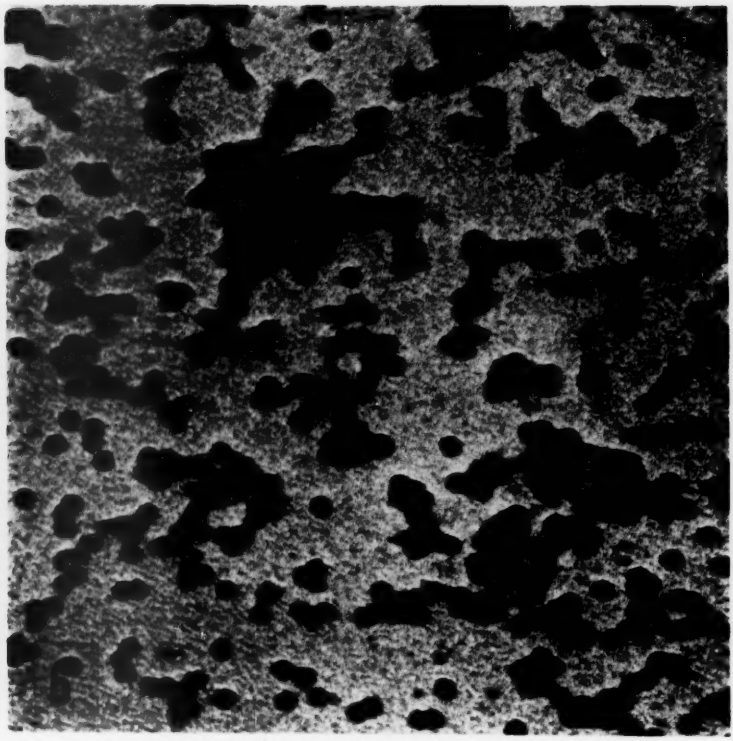

FIG. 6. Poliomyelitis virus, Type 2 (MEF 1), practically unshadowed. A trace of chromium was evaporated on the specimen in order to render the peripheral portions of the particles sufficiently opaque to be imaged by the electron beam. 85000×

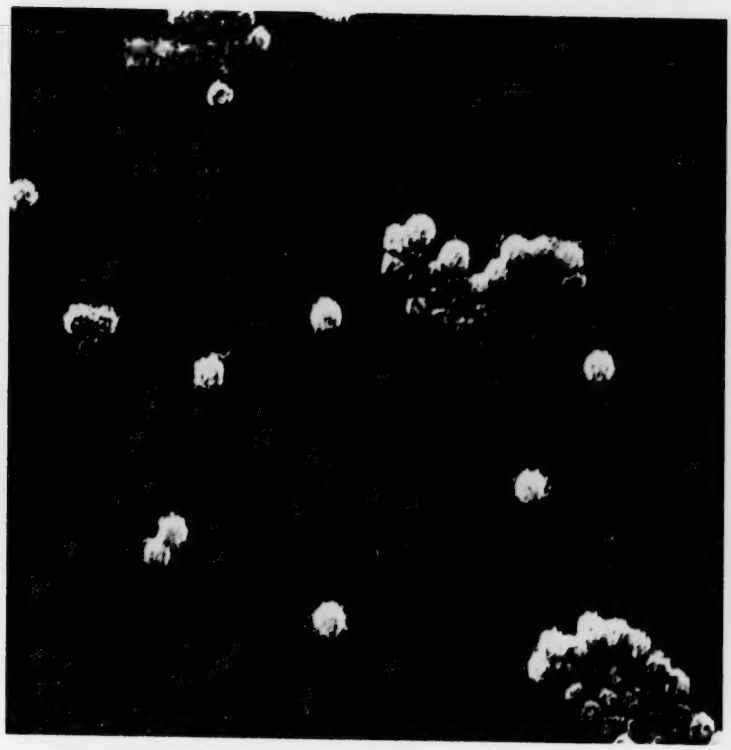

FIG. 7. Poliomyelitis virus, Type 2 (MEF 1), preparation suspended in 1.8 per cent ammonium benzoate, very lightly shadowed with chromium after dehydration. Particles are flattened and the collapsed peripheral membrane structure is visible even on the side toward the shadowing metal source. 180,000×

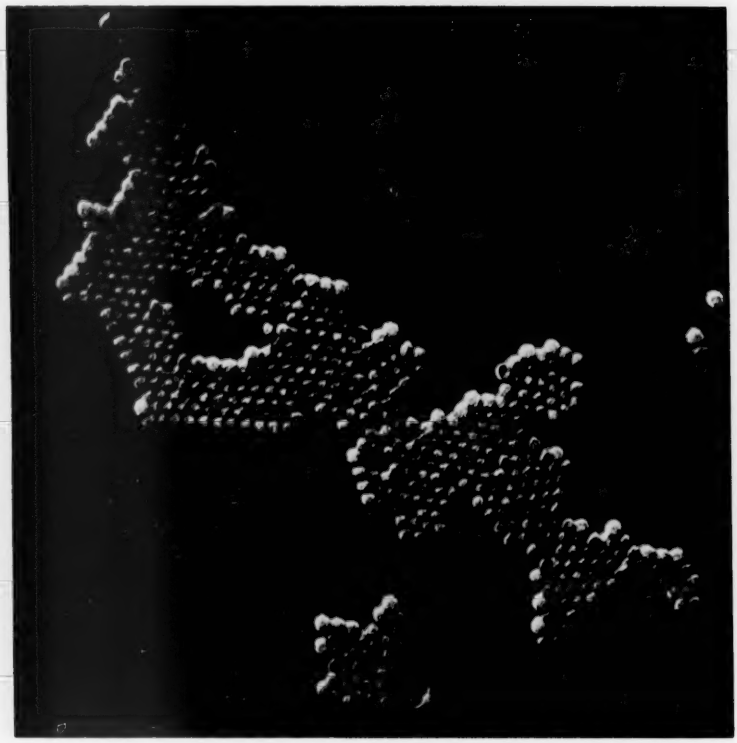

FIG. 8. Poliomyelitis virus, Type *2* (MEF-1). Two-dimensional crystalline array showing uniform hexagonal close packing. 75000x

central portion surrounded by the flattened halo-like membrane. This external collapsed portion is more often than not obscured by the shadowing metal since at most it is only 1 to 2 mμ in thickness. Also of especial note in this micrograph is the variability of the over-all particle size and shape. This could be due to the variability in the size of the dense internal mass of the particles as noted in Figures 3 and 4. It has been our experience that virus particles appreciably dehydrated before shadowing reveal this tendency toward a marked lack of uniformity in size and shape.

Very lightly shadowed preparations of dehydrated specimens, such as described in Figures 3 and 5, have provided an additional means whereby internal detail within poliomyelitis virus particles can be portrayed. The negative print seen in Figure 6 is essentially unshadowed; only a trace of shadowing metal was applied. The magnification is 85,000 times and purified virus (MEF-1 strain), suspended in 1.8 per cent ammonium benzoate solution, was used to prepare the specimen. Evidence of compression in the peripheral portions of the particles is seen in the close array formations and the dense electron-opaque central structure is still revealed. Comparison of these transmission images with those shown in Figure 2 shows that considerably more detail is brought out by the trace of metal coating. In Figure 2 only the dense electron-opaque central portion of the particle is imaged, and it appears in the close arrays that these images are distinctly separated. In contrast, in Figure 6 virtually the entire particle is imaged; the dense central portion is surrounded by the less opaque peripheral structure and the spacing seen in Figure 2 is no longer evident.

The same suspension of purified Type 2 MEF-1 strain virus is used to prepare the specimen in Figure 7. A very light chromium metal coating was evaporated after the dehydration; magnification is 180,000 times. With the light metal coating the collapsed peripheral membrane is visible even on the individual particles and on the side toward the filament location in the shadower. The compression of the outer portions of the particles is seen in the arrays. The shadows, diffuse due to the sparseness of the metal coating, are cast principally by the raised central portions of the virus particles.

In Figure 8 is shown a two-dimensional crystalline array formation of purified Type 2 MEF-1 strain poliomyelitis virus. Such orderly geometric formations (120° angles) are found when relatively concentrated purified virus preparations are filtered through the membranes. They must be shadowed rather heavily in order to withstand the beam in the microscope and although they are often seen in multiple layers, it is exceedingly difficult to obtain well-focused negatives due to thermal vibration in such specimens.

DISCUSSION

The use of collodion as a filtering membrane in the manner described for the preparation and mounting of small pathogenic viruses for electron microscopic examination has distinct practical advantages. The equipment is simple and easily sterilized. The viruses may be handled in saline solutions avoiding the drastic osmotic effects of water and nonphysiological salt solutions.

The moisture, salt, and diffusible material present in the virus droplets placed upon the membranes disappears rapidly, usually within four to five minutes, into the agar below. Evaporation per se is a negligible factor in the method described; comparable droplets placed upon glass slides take one-half hour or more to dry. The virus particles deposited can be dehydrated considerably by allowing prolonged contact of the collodion membrane with the agar after the visible moisture of the droplets has disappeared. In earlier work, "photoflo" and other wetting agents were used in the agar as recommended by Kellenberger, and also in the collodion solution, but experience and practice have indicated to us that they are unnecessary; in fact, the technique as described has yielded more consistent results since discontinuing their use. With animal viruses in the size range up to approximately 100 mμ, the method has proven useful, but with the larger viruses, rickettsiae, and bacteria, conditions become increasingly difficult to control, and distortions and shrinkage due to drying were invariably encountered.

As shown in the electron micrographs, the membranes upon which the specimens are mounted are essentially salt-free. Suspension of the viruses in volatile salt media such as ammonium acetate or benzoate has made no detectable improvement in the quality of the micrographs (compare Figs. 1 and 8). Furthermore, with either nonvolatile or volatile salts the degree of hydration of the virus particles, before subjecting them to the vacuum of the shadower, is controllable to a degree. The critical points appear to be: (i) a "dry" agar surface with the agar itself slightly hypotonic; (ii) a freshly prepared collodion solution; (iii) reasonably rapid transfer of the membrane with the sample from the agar block to the specimen screen, and (iv) a relatively oil-free shadower capable of achieving a vacuum rapidly. The last is undoubtedly a major factor: the pumping system in this laboratory is comprised of a 400-liter-per-second diffusion pump with water-cooled baffles and valving, backed with a Hypervac 23 mechanical pump; stainless-steel wool filters in the rough pumping lines protect the bell jar from oil fog. The system is capable of reaching a vacuum of 10^{-2} mm. mercury in two to three minutes, and better than 10^{-5} mm. mercury in 25 to 30 minutes.

The use of glycerol to alter the permeability of cellular membranes in cell physiological studies, and as a vehicle for stains in cytology, is well known. While the specific action of the glycerol on the surface of the poliomyelitis virus particles is unknown, it is quite obvious that some definite change has occurred. The effect is evident when the virus particles deposited from glycerol media upon a collodion membrane are subjected to high vacuum, Figure 4. However, the chemical and physical characteristics of the particles in solution remain to be determined. Whether or not the biological activities, infectiousness, and antigenicity are retained wholly, or even in part, by the dense internal structures here revealed is not yet known. However, 0.25 per cent glycerol did not interfere with the capacity of crude tissue culture fluids to infect actively growing monkey cell cultures.

In closely packed arrays of poliomyelitis virus it appears that the particles in the outer areas are definitely larger than those within the packed array (Figures 1, 6, 7, and 8). Measurement of six or more particles in rows toward the center of such an array results in an average particle diameter considerably smaller than the measurement obtained from individual particles, sometimes as much as 25 per cent less in larger arrays. Since the poliomyelitis virus particle apparently contains a dense internal structure surrounded by a membrane, it would seem that measurements obtained not only from arrays but especially from individual particles should be interpreted with caution and consideration of the experimental conditions.

Careful examination, in shadowed and unshadowed preparations, of formations in close array and small groups of particles, especially triplets and quartets, suggests that forces other than capillary attraction or surface tension may be involved in bringing about the final orientation as seen in the electron micrographs. With comparatively rigid particles, surface tension and capillarity effects would be expected to give rise to uniform triangular formations where three particles are concerned. Likewise, when four or five particles are drawn together it could be expected that a comparatively uniform parallelogram-like formation would result. Generally, such is not the case as can be seen in Figures 2, 5, and 6. Distortion or compression of the peripheral portions of one or more of the particles in small groups or arrays seems to be the most frequent observation. This could be due, however, to variable size and density of the internal structures of the individual particles, or variations in the degree of hydration. Even in large crystalline arrays (Fig. 8), where the over-all effect is much more uniform, the same type of variations are evident. In Figure 8 there is nevertheless seen a true hexagonal orientation, the adjacent angles all being predominantly 120°. This would suggest that poliomyelitis virus

particles in close array are compressed into a hexagonal shape. Such continued regularity of particle arrangement in three dimensions, which we have seen, would result in a true crystal with the individual particle polyhedral. Electron micrographs of poliomyelitis virus,[11] Coxsackie virus,[2] and human papilloma[6] previously published have revealed similar crystalline array formations.

In earlier work with rabbit papillomatosis[15] and equine encephalomyelitis[16] viruses evidence of internal structure similar to that observed here in poliomyelitis virus (Figs. 2 and 6) was clearly indicated in transmission electron micrographs. More recently, Melnick *et al.*[5,6] have presented evidence that human papilloma virus particles subjected to osmotic shock, or held for prolonged periods in aqueous suspension, appear to possess nucleus-like internal structures. The concept of virus particles having internal bodies surrounded by membranes has been strengthened by the recent developments at Yale[3,9] and Columbia[4] on ultra-thin sections of viruses as they develop within cells. The recent review, "Morphology of viruses,"[1] directs attention to our general lack of knowledge regarding the structure of small viruses and points out that studies such as this must ultimately be correlated with more direct studies of virus and host relationships.

SUMMARY

A method for the preparation of electron microscope specimens is given in which the collodion membranes are formed on agar blocks, used as filtration membranes for the samples, and subsequently mounted on specimen screens for examination.

The present work has led to the hypothesis that the characteristic particles of poliomyelitis virus, approximately 30 mμ in diameter, are "cell-like" structures containing a central body approximately 20μ in diameter and a surrounding membrane.

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STUDIES ON THE PATHOGENESIS OF INFLUENZAL PNEUMONITIS. INTRANASAL VS. INTRAVENOUS INFECTION OF MICE†‡

The respiratory epithelium is the only tissue of the adult mouse that is uniformly susceptible to influenza virus infection. The virus is most effective when administered intranasally[1-4] although pulmonary lesions can be produced less regularly by direct intrathoracic inoculation.[1] Rickard and Francis[18] were the first to demonstrate virus multiplication and pulmonary consolidation after parenteral injection of large doses of PR8 influenza virus, a finding confirmed by Oakley and Warrack[19] for the WS strain. The Henles[6] noted marked variations in the incidence of pneumonitis after massive intravenous and intraperitoneal injections of mouse-adapted influenza viruses. Although each of these investigators observed that the mortality rate in parenterally infected mice was lower than in those infected by the intranasal route, they did not report any basic differences in pulmonary pathology.

During the course of an investigation on virus distribution in mouse tissues, it was found that intravenous injection of the highly pathogenic WS strain of influenza A virus usually produced a nonfatal pneumonitis. The nature of the infectious process, as reflected in the type of virus multiplication and the character of the pulmonary lesions, was deemed to be sufficiently different from classical intranasal infection to warrant a detailed description.

MATERIALS AND METHODS

Male Swiss mice (Webster strain) weighing 10-14 gm. were used in all experiments. The infectivity studies were carried out with the mouse lung-adapted WS strain of influenza A virus using allantoic fluid preparations of the 106th-107th consecutive chick embryo passages. Fluids were obtained from 12-day-old eggs that had been infected 44 hours previously with 0.1 ml. of a 10^{-3} dilution of seed inoculum.

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Mouse inoculation techniques. Intranasal injections were performed under light ether anesthesia by placing 0.05 ml. of the inoculum on the external nares. In experiments involving large numbers of mice, an attempt was made to induce the same level of anesthesia for each animal. Intravenous injections were made in the ventral or dorsal vein of the tail of unanesthetized mice, the usual volume of the inoculum being 0.1 ml. Mice were discarded if there was any question of their not having received a full intranasal or intravenous dose.

Method for obtaining bronchial washings. This method was used to determine the amount of free virus on the respiratory surface of the tracheo-bronchio-alveolar tree. Mice were killed with illuminating gas, the anterior skin reflected laterally and the lungs collapsed by severing the diaphragm just below the xiphoid process. The anterior thoracic cage was then removed without disturbing the visceral pleura. After exposing the trachea a 19-gauge needle on the end of a 1.0-ml. tuberculin syringe was inserted to a point just above the tracheal bifurcation and 0.5 ml. of sterile broth-saline solution introduced into the lungs. This volume of fluid produced equal expansion of all lobes without leakage from the pleural surface. The wash fluid was allowed to remain in the lungs for approximately two minutes before aspiration; each pair of lungs was washed twice with the same fluid. Approximately 95 per cent of the broth-saline solution could be recovered after this procedure; the aspirate was usually slightly cloudy but rarely blood-tinged even in the presence of considerable pulmonary consolidation. It is not certain, of course, that the returns were purely from the respiratory surface unmixed with interstitial fluid, but Fazekas de St. Groth and Donnelly,[8] using a similar technique, have presented evidence for the efficacy of this method.

Infectivity determinations. Lung tissue was emulsified without abrasive in a sufficient volume of broth-saline solution to make a 10 per cent suspension (wet weight). This material was then further diluted in 10-fold steps and 0.1 ml. aliquots of each dilution injected into the allantoic cavities of 4-6 eggs. After 44 hours' incubation the allantoic fluids were harvested and tested individually for their capacity to agglutinate an equal volume of one per cent chicken erythrocytes. In addition to the egg infectivity studies, many of the lung suspensions were tested for hemagglutinin content. This method was less reliable for estimating virus content due to the presence of nonspecific agglutinins at low dilutions which tended to obscure the classical pattern[14] of specific viral hemagglutination. In addition, the direct hemagglutination method is not sufficiently sensitive to detect small quantities of virus since approximately 10^2-10^3 infective doses are required to produce one hemagglutinating unit.

Egg infectivity and hemagglutinin determinations of bronchial washings were made in the same manner, using undiluted fluid as the 10^0 reference point. The results obtained with this material were eminently more satisfactory than those with whole lung suspensions. The bronchial washings contained no particulate matter, provided more uniform suspensions, were invariably free of nonspecific hemagglutinins, and frequently gave higher egg infectivity and hemagglutinin titers than the lung suspensions.

Scoring pulmonary consolidation. The method used was essentially that of Ginsberg and Horsfall[7] by which the degree of lung consolidation is estimated by gross inspection on a scale of 0 to 5+. A percentage rating was then given to each group of similarly treated mice and a mean score (MS_{50}) calculated on the basis of dilution of infective material required to produce a 50 per cent consolidation endpoint.

EXPERIMENTAL RESULTS

Comparative effectiveness of intranasal and intravenous routes of inoculation

The variations in response of mice to intranasal and intravenous inoculation were determined by comparing the amount of WS virus required to produce infection, pulmonary consolidation, and death at the 50 per cent level. Twelve mice were injected by each route with serial 10-fold dilutions of a single allantoic fluid preparation of WS virus having a hemagglutinin titer of 1 : 4096 and an egg infective titer of $10^{9.83}$. Six mice in each group were killed in 4 days and their lungs tested individually for the presence of virus. The incidence of death and the degree of pulmonary consolidation were noted in the remaining animals over a 10-day period.

TABLE 1. THE AMOUNT OF WS VIRUS REQUIRED TO PRODUCE A 50 PER CENT INCIDENCE OF INFECTION, PULMONARY CONSOLIDATION, AND DEATH IN MICE INJECTED INTRANASALLY OR INTRAVENOUSLY

	Log EID_{50}		*I.V./I.N. ratios (Antilog)*
	I.N.	*I.V.*	
ID_{50}*	0.83	6.0	147,000
MS_{50}†	1.83	9.33	31,700,000
LD_{50}†	2.5	>9.83	.....

* In 4 days.
† In 10 days.

The striking differences in susceptibility by the two routes of inoculation are shown in Table 1. As noted, the virus was approximately 100,000 times more infective when administered intranasally. This refractoriness to intravenously injected virus was even more apparent when gross pulmonary lesions and death were considered. In order to produce equivalent degrees of pulmonary consolidation, 31,000,000 more egg infective doses were required by the intravenous route than by intranasal injection. A 50 per cent lethal endpoint was not achieved with the most concentrated intravenous inoculum. Two of six mice died in 48 hours after receiving $10^{9.83}$ EID_{50} intravenously (undoubtedly due to the toxicity of the preparation[8]), but there were no deaths attributable to late pulmonary consolidation.

The fate of the inoculum

It seemed possible that the marked resistance of mice to virus injected intravenously could be due to failure of a sufficient number of infective particles to reach the lung. Or, alternatively, the post-inoculation virus

content of the lung might be high but cells capable of supporting virus multiplication were relatively inaccessible by this route. These questions were explored by comparing the amount of "free" virus recovered in the bronchi and whole lungs at intervals after inoculation. In this manner some estimate was possible of what proportion of the total virus population of the lung could traverse the "blood lung barrier" and find its way to susceptible cells.

As in the previous experiment, various concentrations of virus were injected intranasally or intravenously in groups of 18 mice. At arbitrarily chosen times of 10 minutes and 3 hours after inoculation, when the virus present represented original inoculum, 6 mice were sacrificed and infectivity determinations made of pooled samples of bronchial washings and lung suspensions. The procedure was repeated with the remaining 6 mice in each group 24 hours after inoculation to compare the distribution of actively multiplying virus.

TABLE 2. WS VIRUS CONTENT OF LUNGS AND BRONCHIAL WASHINGS AT INTERVALS AFTER INTRANASAL AND INTRAVENOUS INFECTION

Route	Dose/mouse (Log EID_{50})	Infectivity ratios = Log EID_{50} Whole lung/Bronchi		
		10 min.	3 hr.	24 hr.
I.V.	9.83	6.0/0.75	4.37/2.5	5.5/6.17
	7.83	4.5/1.5	3.0/0	4.33/4.5
	6.83	4.67/0*	1.33/0	4.25/5.5
	5.83	4.25/0	0/0	0.67/0
I.N.	3.83	2.75/2.5	1.23/1.75	7.5/7.0
	1.83	0/0	0/0	4.67/3.5

* 0 = $<10^{0.5}$.

The results are summarized in Table 2. The proportion of the intravenously injected virus recovered from whole lung in 10 minutes varied with the infecting dose, ranging from 2.6 per cent of the most dilute inoculum to 0.01 per cent of the most concentrated. By the 3d hour after intravenous injection a considerable decline in the infective virus content of whole lung was noted at each dose level. Relatively few infective particles were found in bronchial washings at 10 minutes and 3 hours and only in the most heavily exposed mice, representing but a small fraction of the total lung virus. In the animals that received $10^{6.83}$ EID_{50}, no virus could be demonstrated in bronchial washings, but its presence there was attested to

by the appearance of bronchial virus in moderately high concentration at 24 hours. Whereas the post-inoculation samples showed a preponderance of virus located extrabronchially, when multiplication ensued, the bronchial titers exceeded those of whole lung. Undoubtedly, the presence of small amounts of bronchial virus could not be detected due to the virus-binding capacity of the cells. This is most clearly demonstrated by intranasal inoculation which resulted in rapid absorption of virus by respiratory epithelium leaving less than 10 per cent of the inoculum free in the bronchi after 10 minutes. As opposed to intravenous injection, the whole lung/bronchial ratios of intranasally administered virus were about equal.

There were no significant differences in the recovery rates of intravenously injected virus when the volume of the inoculum was increased to as much as two ml.

It would appear, therefore, that the relative innocuousness of intravenously injected virus is due, at least in part, to its inability to reach susceptible cells in adequate concentration to initiate infection.

Virus growth curves

The interrelationship of pathogenicity and multiplication rate of influenza viruses has been stressed by many investigators. Different strains vary in their capacity to produce transmissible pneumonitis after intranasal injection depending upon their multiplication potentials which, in turn, are related to their degree of adaptation.[21] Rapidly proliferating strains cause fatal pneumonitis with inocula of low infectivity, whereas avirulent, unadapted strains grow poorly and do not produce pulmonary consolidation unless massive doses are injected.[19] Multiplication of adapted strains may also be limited with very dilute inocula, in which case the onset of pulmonary consolidation is correspondingly delayed.[7] In the last analysis, probably the most important factor in pathogenicity is the total amount of virus that the respiratory epithelium is exposed to at any one time, regardless of whether this be from newly formed virus or weakly infective inoculated virus. This has been most clearly demonstrated by Ginsberg[6] who found that in vaccinated mice immunity could be overcome and extensive pneumonitis produced with massive intranasal inoculations, in spite of the absence of a detectable cycle of virus replication.

An example of the influence of the infecting dose on the multiplication rate of intranasally injected WS virus is shown in Figure 1. When the inoculum contained $10^{3.83}$ EID_{50}, the maximum lung and bronchial concentrations of infective virus were achieved within 24 hours and all the mice died in 5 to 7 days with complete pulmonary consolidation. On the other

hand, intranasal inoculation of $10^{1.83}$ EID_{50} resulted in a slower increase of virus, generally lower titers late in the course of infection, and an over-all incidence of only 50 per cent lung lesions. Also noteworthy is the fact that the bronchial titers frequently exceeded the total lung titers in the same mice, indicating that a considerable amount of virus was "free" in the bronchial secretions and that the cells were "saturated."

Returning for a moment to Table 1, it can be seen that a more dilute intranasal inoculum ($10^{0.83}$ EID_{50}) retained its infectivity but completely lost its pathogenicity. By the intravenous route, on the other hand, greater

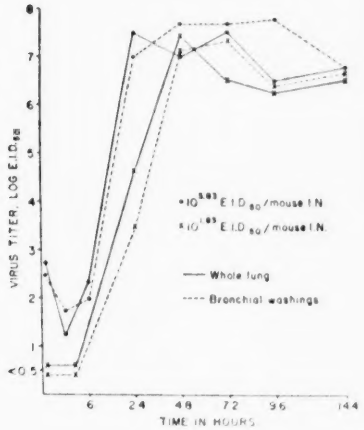

FIG. 1.

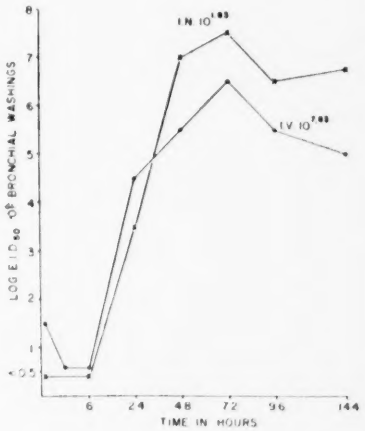

FIG. 2.

FIG. 1. The relation of multiplication potential to dose of intranasally administered WS virus.

FIG. 2. WS virus growth curves after intranasal and intravenous inoculation.

than a 1000-fold increase in the ID_{50} level was required to produce 50 per cent consolidation and an LD_{50} concentration was unattainable. These data suggested, that, regardless of the amount of virus reaching the respiratory epithelium after intravenous injection, it was severely limited in its proliferative potential, both as to rate and extent of multiplication.

Support for this hypothesis was obtained by examining in detail the virus growth curves after intravenous injection of various concentrations of WS virus. As seen in Table 3, there was little difference in production of infective virus over a wide intravenous dose range and, most strikingly, the titers rarely exceeded $10^{6.5}$ EID_{50} even with the heaviest inoculum. Also, there was no evidence of significant virus multiplication outside of the

respiratory epithelium for, as was the case with intranasal infection, the virus content of the bronchial washings was usually greater than that of whole lung in the same mice. This, incidentally, would appear to be presumptive evidence that bronchial epithelial cells are infected by intravenous as well as intranasal injection.

Bronchial growth curves are presented in Figure 2 to show more clearly the comparative response of mice to a small intranasal and a large intravenous dose of WS virus. It is apparent that the mechanism of infection is quite different, not only in regard to the invasiveness of virus by the two routes, but also in the essential dissimilarity of virus growth.

TABLE 3. DAILY WHOLE LUNG/BRONCHIAL WS VIRUS TITERS (—LOG EID_{50}) AFTER INTRAVENOUS INJECTION

Log I.V. dose	*Days after injection*					
	1	*2*	*3*	*4*	*5*	*7*
9.83	5.5/6.17	5.5/6.5	6.0/6.29	6.25/7.0	5.5/6.4	4.75/4.5
7.83	4.33/4.5	5.5/5.5	5.78/6.5	4.75/5.5	.....	4.33/5.5
6.83	4.25/5.5	4.33/4.25	4.5/5.5	5.5/5.33		
5.83	0.67/0*	0/0	4.33/5.25	3.76/4.33		
4.83	0/0	0/0	0/0	0/0		

*0 = $<10^{0.5}$.

Hemagglutinin-infectivity ratios

One possibility that required consideration was that intravenous infection results in an entirely different mode of virus replication involving the production of noninfectious particles, the so-called "incomplete virus" of von Magnus.[11] Such a cycle of influenza virus multiplication has been demonstrated in insusceptible tissues like mouse brain after direct inoculation of very large doses of the PR8 strain.[15] Since mouse lung also exhibited a relatively poor response to massive parenteral doses of virus, further studies were carried out to determine whether noninfectious hemagglutinins appeared at any stage after intravenous infection.

An allantoic fluid preparation of WS virus was used that contained approximately $10^{5.5}$ EID_{50} for each unit of hemagglutinin, a ratio which is in the usually accepted range for highly infective influenza virus. This material was injected undiluted into 24 mice by the intravenous route and individual assays made of the hemagglutinin and egg infective titers of both the bronchial washings and whole lung suspensions of 4 mice at daily intervals thereafter. Hemagglutination titrations of all specimens were carried

out in citrate-saline solution after treatment with receptor-destroying enzyme in order to inactivate virus inhibitors.

As shown in Table 4, the expected ratios of hemagglutinating to infective virus were obtained throughout the 6-day experimental period. It appears reasonable to assume, therefore, that only fully infective particles are being produced in response to intravenous infection. Similar ratios were also obtained after intranasal injection. Furthermore, these experiments provide additional evidence that the differences in the virus growth curves after intravenous infection are not due to significant extrabronchial multiplication of either infective or "incomplete" virus.

TABLE 4. HEMAGGLUTININ/INFECTIVITY RATIOS AFTER INTRAVENOUS INJECTION OF UNDILUTED WS VIRUS

	Day				
	1	*2*	*3*	*4*	*6*
Whole lung	$0/10^{0.5}$	$0/10^{4.2}$	$2/10^{6.05}$	$0/10^{6.5}$	$3.2/10^{6.8}$
Bronchi	$0/10^{3.0}$	$0/10^{5.75}$	$2/10^{7}$	$6.4/10^{7.88}$	$8/10^{8.0}$

The values are the reciprocal geometric mean.
HA/EID$_{50}$ titers of 4 mice. $0 = <2$.

Although the hemagglutinin titer may provide a more accurate means for following the course of well-established infections, it is quite apparent that this method is insufficiently sensitive to detect the presence of low grade infections. Nevertheless, most of the detailed studies of influenza virus infection of mouse lung that have been done in the recent past have used viral hemagglutinins as the index of infection. For example, in the exhaustive series of experiments reported by Fazekas de St. Groth and Donnelly[8] there was no hemagglutinin detectable in the lung after large intraperitoneal or subcutaneous injections of the Melbourne A strain, leading these authors to conclude that "active virus given by extrarespiratory routes is as incapable of causing infection as inactivated vaccines given by any route."

Serial intravenous passage

In their studies on influenza virus toxicity the Henles[9] found considerable variation in the frequency with which late pulmonary consolidation could be produced after parenteral injection of mouse-adapted strains. Since it was conceivable that these strain differences might have been related to degree of adaptation, an attempt was made to augment the intravenous pathogenicity of WS virus by serial passage in the hope that a more

virulent form would arise. Presumably, this might occur by mutation or selection of a variant with greater invasive and proliferative potential by the intravenous route of inoculation. The series was initiated by injecting 10 mice intravenously with 0.1 ml. of undiluted WS allantoic fluid. After 4 days 5 of the animals were sacrificed and their bronchial washings collected, titered, and pooled. This material was then immediately injected intravenously into 10 new mice and the cycle repeated thereafter at 4-day intervals. The incidence of death and the degree of pulmonary consolidation over a 10-day period were recorded in the remaining 5 mice of each group.

No deaths occurred and only minimal pulmonary consolidation was noted during the first 9 passages. Deaths were occasionally encountered in subsequent passages, but the mortality rate did not increase consistently. The degree of pulmonary consolidation was also more marked in the later passages but was almost always sporadic and rarely exceeded an over-all score of 50 per cent, occasionally reverting to less than 10 per cent for no apparent reason. Successive transfer did not result in increasing infectivity and hemagglutinin titers of the bronchial washings nor was there any definite correlation between virus content of the lungs and extent of pneumonitis. The experiment was terminated at the 28th consecutive intravenous passage at which time the virus was somewhat more pathogenic but exhibited no alteration in its multiplication potential in mouse lung by either the intravenous or intranasal route.

The Taylor phenomenon

As previously mentioned, an important limiting factor in the intranasal pathogenicity of any virus strain is the dose of the inoculum. Small doses of virus undergo a reproductive cycle characterized by a slower rate of increase and invariably lower titers than do lethal inocula. However, at 48 hours after infection the lungs of surviving mice may contain more than 1,000,000 infective virus particles. The most succinct expression of this paradox is contained in a paper by Taylor:[39] ". . . the lungs of one of these mice contained sufficient virus to infect fatally 70,000 normal mice, yet the mouse whose lungs harbored this enormous amount of virus in some way managed to master the infection and survive. Nor did the virus further increase after it reached a maximum at 48 hours following inoculation." Somewhat by accident Taylor[39] discovered that this phenomenon is related to mechanical bronchogenic dissemination and the capacity of the host to localize the sublethal infection. Simple intranasal instillation of sterile water, saline solution, broth, or 10 per cent normal serum four days after a sublethal inoculation "produced a sharp rise in the virus content of the

lungs followed by death in 3 to 8 days." Pathological lesions in these mice were identical to those seen in animals receiving a lethal intranasal injection. Similar observations had also been made by Straub.[17]

The Taylor phenomenon afforded an additional means of comparing sublethal intranasal and intravenous pulmonary infections. A group of 20 mice was injected intranasally with $10^{1.83}$ EID_{50} of WS virus and an equal number with $10^{7.83}$ EID_{50} intravenously. These doses of virus by themselves did not cause death and produced roughly equivalent bronchial titers. Four days after injection each of these 40 mice was given 0.05 ml. of sterile broth-saline solution intranasally. All 20 of the mice originally infected by the intranasal route died with complete pulmonary consolidation between 2 and 8 days after the second inoculation. On the other hand, only 5 of the 20 intravenously infected mice succumbed from 3 to 7 days after intranasal instillation of broth-saline solution. It would appear, therefore, that intravenously administered virus is in a less advantageous position to be disseminated by the bronchogenic route.

Interfering action of intravenously administered virus

Rickard and Francis[13] have presented evidence of a rapidly developing resistance to intranasal PR8 virus infection in mice previously inoculated intraperitoneally with live homologous virus. This protective effect was maximal when the interval to challenge was two days, during a time when circulating antibody could not be detected. Oakley and Warrack[12] were unable to duplicate these results with either PR8 or WS virus but entertained the possibility that their preliminary intraperitoneal injections were too small to confer resistance.

The results of the following experiment support the findings of Rickard and Francis.[13] Four groups of 20 mice were injected with normal allantoic fluid (NAF) or sublethal doses of WS virus, as follows: Group I—NAF 0.05 ml. intranasally; Group II—NAF 0.1 ml intravenously; Group III—$10^{1.83}$ EID_{50} of WS virus intranasally; and Group IV—$10^{7.83}$ EID_{50} of WS virus intravenously. Two days after these preliminary injections all 80 animals were challenged with 50 LD_{50} of WS virus by the intranasal route. As shown in Table 5 all but 1 of the 60 mice in Groups I, II, and III died with complete pulmonary consolidation. On the other hand, 60 per cent of the mice that had received a preliminary intravenous virus inoculation survived the challenge injection, in spite of the fact that extensive pulmonary consolidation was noted at the end of the 10-day experimental period.

It would appear, therefore, that the interfering action of intravenously injected virus represents an additional distinguishing feature from classical intranasal infection.

Pathology

Credit for the earliest and most concise description of the morphology and pathogenesis of murine influenza virus pneumonitis belongs to Straub.[17, 18] He states that following intranasal infection "the first localisation of this process (gross lesion) is often found near the hilum of one or more lobes; later on entire lobes or almost the whole of both lungs except the edges may be affected." The gross morphological changes were not considered to be peculiar to influenza virus infection but many of the microscopic lesions appeared to be quite specific reactions to this organism. His most pertinent observation was that experimental influenza infection in the mouse is primarily a disease of bronchial and bronchiolar epithelium.

TABLE 5. RESISTANCE TO INTRANASAL CHALLENGE OF LD_{50} OF WS VIRUS TWO DAYS AFTER PRELIMINARY INJECTION

	Interfering virus			
Group	*Route*	*Dose (EID_{50})*	*Mortality*	*% Lung lesions*
I	I.N.	0 (NAF)	20/20	100
II	I.V.	0 (NAF)	19/20	99
III	I.N.	$10^{7.8}$	20/20	100
IV	I.V.	$10^{7.8}$	8/20	80

Sequentially, there is destruction of epithelial cells which desquamate in 2 or 3 days followed by replacement with a fibrinoid material poor in inflammatory cells. Even the terminal bronchioles, which in the mouse are lined with cuboidal epithelium to their junction with alveolar sacs, are involved in this process but the alveolar cells themselves are spared. Parenthetically, a true hyaline membrane has not been noted.[10] Associated with these changes are peribronchial edema, collapse of alveolar segments with compensatory emphysema in uninvolved areas, interstitial edema and hemorrhage, and varying degrees of infiltration with polymorphonuclear leukocytes. The final gross appearance of plum-colored consolidation is due, therefore, to atelectasis, the extent of the lesions being dependent upon the degree of bronchiolar involvement. Death ensues only when most of the pulmonary parenchyma is involved, varying with the virus dose from 3 to as long as 10 days after intranasal injection. Somewhat more detailed descriptions of certain aspects of the pathogenicity of this disease, particularly the early cytoplasmic and ciliary changes in epithelial cells, are contained in the reports of Loosli[10] and Harford and Hamlin.[7]

Of the greatest interest, perhaps, is Straub's observation that the reparative process may begin as early as the 5th day even in fatal infections. Proliferation of new bronchial and bronchiolar epithelium is seen at this time which is low cuboidal or squamous in appearance, closely resembling the lesions described by Winternitz *et al.*[22] in pandemic human influenza. Mice surviving the infection may show an extraordinary picture several weeks after inoculation. The involved portions of the lung contain nests of squamous epithelial cells with many mitotic figures, not only in the bronchioles but also proliferating downward to fill the alveoli. However, this metaplastic squamous epithelium does not invade interalveolar spaces, and the alveolar septa themselves appear to be well preserved.

In the present studies the pathological changes in the lungs following intranasal injection of WS virus were essentially similar to those described above. *Macroscopically*, plum-colored consolidation was noted in the perihilar areas in 2 to 4 days, depending on the virus concentration of the inoculum. Mice killed thereafter usually showed more extensive lesions spreading toward the periphery but even the lungs of those mice dead of infection usually exhibited a narrow rim of uninvolved lung tissue. The early *microscopic* lesions in intranasally injected mice were those of bronchitis with progressive desquamation of epithelium, accompanied by peribronchial edema and more extensive peribronchitis and intraluminal exudate than described by Straub[17] and Loosli.[16] This process, particularly with small intranasal doses of virus, began in the large bronchi (Fig. 3) and gradually spread to involve the bronchioles. Replacement of columnar epithelium by squamous or low cuboidal cells was noted as early as the 4th day in the large bronchi (Fig. 4) and 1 to 3 days later in the bronchioles.

In marked contrast, the *gross* pathological findings in intravenously infected mice were characterized by peripheral, frequently wedge-shaped pulmonary lesions with hilar sparing. These focal areas of plum-colored consolidation could be found as early as 3 days after infection and were most extensive by the 4th or 5th day. The lesions usually remained localized and ordinarily did not progress to involve the entire lung as was the case with intranasal infection. Most frequently affected were the right middle lobe, the apical segment of the left lung, and the subcardiac lobe of the right lung, in that order. The earliest *microscopic* lesions in intravenously infected mice occurred in the small and medium-sized blood vessels of the lungs, both arteries and veins. Extensive perivascular infiltration with polymorphonuclear and mononuclear leukocytes was noted 2 to 3 days after injection, remaining stationary with little progression or resolution for at least several weeks (Fig. 5). Only rarely were inflammatory cells seen invading blood vells walls and there was no destruction or proliferation of

vascular endothelium. Thromboses were uncommon. Destruction of respiratory epithelium was noted by the 3d or 4th day after intravenous infection, usually focal in distribution and limited to the terminal bronchioles. Unlike intranasal infection the larger bronchioles and bronchi were never involved and the degree of peribronchiolar inflammation was minimal. Consolidation, hemorrhage, edema, and collapse of alveoli occurred in patchy segments, generally at the periphery of the lung and not unusually involving the visceral pleura. Straw-colored pleural effusions were occasionally seen with intravenous but not intranasal infections.

Squamous metaplasia of the bronchiolar epithelium appeared on the 4th or 5th day after intravenous infection, becoming increasingly more prominent thereafter, and was far out of proportion to the rather minimal degree of bronchiolar destruction that preceded it. The lungs of mice killed two weeks after intravenous infection presented a striking picture (Fig. 6). The periarteritis had not subsided and squamous epithelial cells with numerous mitotic figures filled nests of alveoli. By the 23d day the extent of proliferative epithelialization took on an appearance suggestive of bronchogenic carcinoma of the squamous cell type, but closer examination revealed that the interalveolar spaces were not invaded and the basic architecture of the lung was preserved. There was no desmoplastic reaction in the epithelial cell masses and the degree of interstitial fibrosis was minimal. Very little resolution of these lesions was noted as long as three months after infection.

On the basis of the superficial resemblance of these epithelial changes to neoplasia[17, 18] an attempt was made to transplant lung tissue of intravenously infected mice to the subcutaneous space of homologous hosts. Many of the homografts survived for several weeks but did not exhibit true epithelial proliferation and could not be retransplanted.

The gross and microscopic pathological changes in the lungs of the intravenous serial passage mice were similar to those described above but were generally more extensive. Infected allantoic fluid diluted beyond 10^{-3} or normal allantoic fluid injected intravenously produced no vascular or bronchiolar lesions.

The lungs of mice that survived a challenge intranasal injection of WS virus given two days after an intravenous interfering dose showed the histological effects of intranasal infection plus the perivasculitis of intravenous infection. Grossly and microscopically the consolidative process ten days after intranasal challenge was confined to the hilar and peribronchial areas, almost as if the centrifugal spread of the lesions had been arrested at the terminal bronchioles.

FIG. 3. Intranasal infection. Marked inflammatory response in a major bronchus four days after intranasal inoculation of $10^{5.8}$ EID_{50} of WS virus. Hematoxylin-eosin, ×90.

FIG. 4. Intranasal infection. Higher power (×410) view of the wall of the bronchus in Figure 3 showing the thickened cuboidal metaplastic epithelium. Note the lack of perivasculitis of the adjacent arteriole.

FIG. 5. Intravenous infection. Marked perivasculitis, well-preserved columnar bronchial epithelium and early squamous metaplasia six days after infection. The visceral pleura is also infiltrated with inflammatory cells. Hematoxylin-eosin, x130.

FIG. 6. Intravenous infection. Masses of squamous epithelial cells filling nests of alveoli in the lung of a mouse that survived 14 days. Note the fairly well-preserved bronchial epithelium and residual perivasculitis. Hematoxylin-eosin, x130.

DISCUSSION

Regardless of whether virus is administered intranasally or intravenously, the pathogenesis of influenzal pneumonitis is clearly related to bronchiolitis. However, the rate of virus multiplication and the severity of the disease depend, to a large extent, on the route and virus concentration of the inoculum. Thus, the response to intranasal infection may take three forms depending on the degree of exposure. With large infective doses the bronchi and bronchioles are probably involved simultaneously, the virus multiplying at all sites of primary infection and producing maximum titers within 24 hours. This fulminating infection is accompanied by generalized, uniform destruction of respiratory epithelium and early atelectasis, consolidation, and death. An alternative course may be seen with smaller doses of virus in which the infection is probably confined at first to the upper respiratory tract and major bronchi resulting in a generally slower rate of virus proliferation. Due to involvement initially of fewer cells, maximum titers are not obtained until 48 hours after injection and the occurrence of lung lesions and death is correspondingly delayed. Thirdly, sublethal doses produce relatively few new infective particles, presumably because the infectious process is localized to the major bronchi; the majority of bronchioles are spared, the incidence of atelectatic and pneumonitic lesions is low, and the host, therefore, survives. It is of interest that this capacity to resist a low-grade infection is largely a matter of lessened mechanical dissemination of virus, since simple intranasal instillation of bland fluids can convert the benign course to a fulminating pneumonitis. Notwithstanding these differences in dose response, influenzal pneumonitis produced by intranasal injection is essentially a descending bronchogenic infection.

In contrast, infection by the intravenous route appears to be primarily a focal disease of terminal bronchioles. By and large the virus has no way of ascending the bronchial tree and in only a limited number of animals can disseminated infection be produced by intranasal instillation of sterile broth. Since the infectious process is generally confined to the bronchiolar epithelium, the resulting virus titers are lower than after intranasal infections, rarely attaining the critical levels of a lethal infection. Although severely limited, the multiplication potential of the virus and the degree of pulmonary consolidation depend to some extent on the number of infective particles that reach susceptible cells by the intravenous route which, in turn, is a function of dose of inoculum. Unlike intranasal infection, the range between infective and pathogenetic doses of intravenously injected virus is extremely wide.

One possible explanation of the limited susceptibility of lung tissue to intravenous infection would be that virus replication takes place on the extrarespiratory surface of the capillary-alveolar membrane. An analogy to such a situation can be found in the differential susceptibility of the two sides of the chorio-allantoic membrane of the chick embryo. Fulton and Isaacs[8] clearly demonstrated that the chorionic surface is capable of supporting only a single cycle of influenza virus multiplication and the amount of antigen produced in these cells is dependent on the infecting dose. Significantly, they also found that virus did not penetrate to the susceptible allantoic cells. Of interest also are the findings of Burnet and Fraser[2] that certain strains of virus inoculated intravenously or onto the chorionic surface of the chick embryo chorio-allantois are disseminated to the brain, resulting in a hemorrhagic encephalitis. Previous inoculation of homologous virus by the allantoic route interfered with the development of cerebral hemorrhages, and the embryos survived. These authors suggested that virus multiplication and interference take place in the capillary endothelial cells of the brain. However, the evidence in the present studies appears to be fairly conclusive that multiplication of intravenously administered virus is confined to the bronchiolar epithelial cells of the mouse. Except for the immediate post-inoculation period the virus content of bronchial washings always equalled or exceeded that of whole lung, indicating that little if any infective virus remained in the interstitium or capillaries. In addition, the pathologic lesions were those of bronchiolitis rather than interstitial pneumonia, and perivasculitis without endothelial involvement.

Assuming, therefore, that only the respiratory epithelium of the lung can serve as substrate for influenza virus multiplication, there appear to be three possible routes by which the virus can cross the "barrier" from blood stream to terminal bronchiolar cells. The first is that rupture of submucosal capillaries may spill virus into the bronchial tree. This explanation seems least likely because large volumes of inocula, up to two ml. intravenously, did not enhance pulmonary infectivity, and no extravasation of blood was seen in sections taken immediately after injection. Of greater likelihood is that small blood vessels are permeable to small quantities of virus adherent to their walls. This mode of penetration would be more consistent with the invariable pathological finding of perivasculitis. Having found its way into the interstitial tissues the virus may then invade bronchiolar epithelium by the "back door," that is, the extrarespiratory surface of the cells. An equally tenable explanation, however, would be that virus diffuses across the capillary-alveolar membrane with reflux upward to infect adjacent epithelial cells at their respiratory surface. This last hypothesis would perhaps best

explain the primary infection of terminal bronchioles and the sparing of larger branches.

SUMMARY

The pathogenesis of murine influenzal pneumonitis was studied by comparing the host response to intranasal and intravenous infections with the mouse-adapted WS strain of influenza A virus. Striking differences were found in infectivity, pulmonary consolidation, and death by the two routes of inoculation, as well as post-inoculation distribution of virus in lungs and bronchi, virus multiplication potentials, capacity of the host to localize low-grade infections, and interference phenomenon. The primary pathological findings in either type of infection were early destruction of respiratory epithelium and subsequent metaplastic regeneration. However, the distribution of the lung lesions was quite different. After intranasal infection the major air passages were involved initially, progressing to extensive bronchitis, atalectasis, diffuse pulmonary consolidation, and death; whereas, intravenous infection was essentially a benign, focal disease of terminal bronchioles. It is concluded that the severity of the pneumonitic process is a function of the extent to which the total mass of respiratory epithelium is involved, which is, in turn, related to mechanical bronchogenic dissemination of virus.

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A CORRELATION OF EARLY RADIATION CHANGES IN LYMPHATIC TISSUES WITH ANTITOXIN-PRODUCING ABILITY

Investigations concerning the sites of antibody formation have been underway for a number of years. Due to the difficulty that has been encountered, many approaches have been used. These have included surgical removal of portions of the organism, tissue culture, lymphatic canulation, attempts to overload the phagocytic system, studies of antibody content of local sites of immunization, partial shielding from radiation effects, and correlation of histology with antibody rise.

Recently Hale and Stoner[13, 20] by intraocular transplantation techniques have demonstrated the production of antibodies by transplants of spleen, lymph node, thymus, and Peyer's patch in the anterior chamber of mice eyes. Oakley and co-workers[21, 22] have demonstrated antibody production by local sites of injection of alum-precipitated diphtheria and tetanus toxoid by transplanting what is apparently from their gross description a granuloma. This is consistent with the findings of Hartley[18] and his illustration of a local macrophage reaction at injection sites.

This evidence has resulted in three concepts of the cells of origin of antibodies. It is interesting that these concepts are not necessarily exclusive of one another. One group supported primarily by Dougherty, Chase, and White[5, 6, 27] and earlier by Harris and Harris[15] and co-workers[7, 14] suggest that the lymphocytes form or transport antibodies[28] and may even be responsible for maintenance of normal serum globulin. Habel,[12] however, in 1949 still felt that the immunological evidence for the lymphocyte production of antibodies should be considered incomplete.

More convincing evidence accumulated by Fagraeus,[10] Keuning and van der Slikke,[19] Darcy,[8] and Ehrich and co-workers,[9] and others[2] suggests the plasma cell as either the site of origin or means of transportation of antibodies. Fagraeus, however, has pointed out the importance of a more undifferentiated cell, the "transitional" cell, by morphological studies.

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Antibody production by means of the reticulo-endothelial system has long been suggested, though, as stated by Roberts in 1953,[24] the conclusive experiment has not been done. Localization of particulate and dye combined antigens in cells of the reticulo-endothelial system has been well established by Sabin,[25] Gabrieli,[11] and others. Utilizing fluorescent conjugated antibodies, Coons and co-workers[8, 17, 18] have illustrated the localization of antigens in macrophages of the spleen, lymph nodes, in Kupfer cells, lung septal cells, and in endothelium. It would seem from their work that the only cell type which invariably fixes antigen is the reticulo-endothelial phagocyte, even at low dosage and for a long period (75 days). Antigen was detectable in lymphocytes for only one week. The work of Hartley[16] and Oakley[22] re-emphasizes that phagocytic cells are capable of antibody production.

In this report* we have[13] correlated the morphological changes in lymphatic tissues with the ability of the animal to produce tetanus antitoxin following irradiation and[20] correlated the change in orphology of the thymus of old mice with the increase in ability of thymic transplants from old mice to produce antitoxin. The present work points out the ability of the cells of the reticuloendothelial and/or phagocytic system to produce antibodies.

METHODS AND MATERIALS

Highly inbred Swiss albino, infection-free mice maintained in our own colony (W.M.H.) were used in each of these experiments. Animals utilized for determining the antitoxin-producing ability were given 0.05 ml. alum-precipitated tetanus toxoid subcutaneously as their primary stimulus 21 days prior to the secondary stimulus of 0.05 ml. fluid tetanus toxoid. The mice were one to two and a half months old with an equal distribution of age ranges in each group. Blood was collected ten days following the secondary stimulus and the pooled serum titrated, in the manner previously described.[13]

Animals utilized for the histological study of the radiation changes were from the same colony and all approximately two months old. The animals were autopsied at time intervals following irradiation. Tissues were fixed in Zenker's acetate, sectioned by the paraffin method and stained with H & E and Giemsa.

Whole-body radiation was delivered by a cobalt-60 gamma source. The dosage, 650 rep, is less than the $L.D._{90}$ 30 days which is 750 rep for our animals.

EXPERIMENTAL

The first experiment was designed to test the effect of gamma irradiation upon the antitoxin response to the secondary stimulus. The mice were divided into eight groups of thirty mice each. One group was irradiated at

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each of the following periods: two days, one day, twelve hours, six hours, and one hour prior to the secondary stimulus and six hours, one day, and three days after the secondary stimulus. The animals were bled ten days following the intra-peritoneal secondary stimulus, and titrations were performed as previously described.[13] The results are shown in Chart 1. For the histological studies two animals were autopsied at twelve periods following irradiation as follows: thirty minutes, one hour, and hourly intervals thereafter for ten hours. Tissues were fixed in Zenker's acetate, paraffin sectioned, and stained with H & E and Giemsa.

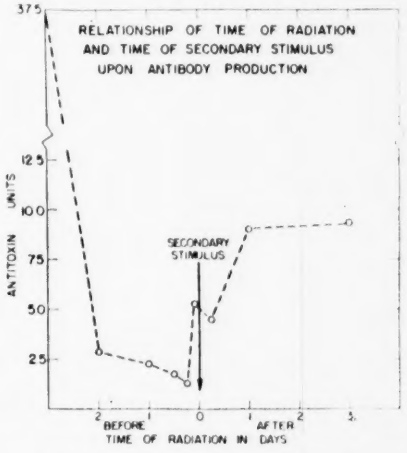

CHART 1. The primary stimulus was given by injecting 0.05 ml. of alum-precipitated tetanus toxoid subcutaneously.

The secondary stimulus was due to the injection of 0.05 ml. fluid toxoid intra-abdominally.

The interval between the two stimuli was 28 days.

The amount of antibody produced, as shown in Chart 1, indicates that there is relatively little difference between irradiation given two days before the secondary stimulus and irradiation given six hours before the secondary stimulus. Irradiation given six hours prior to the secondary stimulus results in the minimal response of the animals. Irradiation at one hour prior to the secondary stimulus results in a four-fold increase over the amount of antibodies produced by the group irradiated six hours before. This would indicate that the one- to six-hour period is a critical time during which metabolic changes are taking place in the cells which produce antibodies. This, however, is dependent upon the assumption that the reaction stimulated by the antigen takes place immediately, or completely within the first six hours. Since it is believed that antibodies are not produced in response to a secondary stimulus within the first six hours, we may assume that morphological changes occurring during this six-hour period are of negligible effect upon antibody production. It appears then that it is the early changes occurring during this period and becoming more marked within the next several hours that are of fundamental importance in determining those cells which produce antibodies. It is important also to point out that

metabolic changes within the cell undoubtedly precede morphological changes.

The morphological studies of lymphatic tissues, proven to produce antibodies, during this early post-irradiation period, should, therefore, indicate important changes taking place within cells that produce antibodies. Figure 1 illustrates early changes taking place within the axillary lymph node of a mouse one hour after 650 rep. Focally there is necrosis of lymphocytes and plasma cells as demonstrated by chromatin clumping and karyorrhexis. Figure 2 illustrates the progression of this necrosis at seven hours after irradiation. There are few recognizable mature lymphocytes remaining. The morphologically earlier changes of nuclear swelling and karyolysis are occurring within more undifferentiated cells, the phagocytic or reticuloendothelial cells.

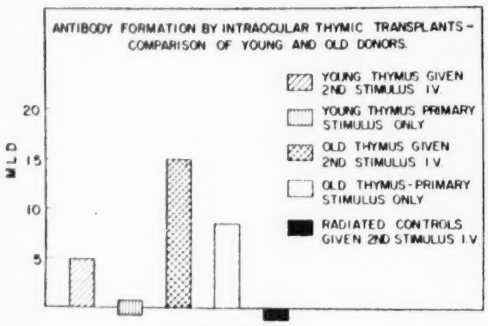
ANTIBODY FORMATION BY INTRAOCULAR THYMIC TRANSPLANTS – COMPARISON OF YOUNG AND OLD DONORS.
YOUNG THYMUS GIVEN 2ND STIMULUS I.V.
YOUNG THYMUS PRIMARY STIMULUS ONLY
OLD THYMUS GIVEN 2ND STIMULUS I.V.
OLD THYMUS-PRIMARY STIMULUS ONLY
RADIATED CONTROLS GIVEN 2ND STIMULUS I.V.
M.L.D.
20
15
10
5

CHART 2.

Figure 3, showing the spleen at three hours after irradiation, illustrates again the marked early changes occurring in the lymphocytes and plasma cells and the earlier degenerative changes occurring in cells of the phagocytic system. This is more dramatically illustrated in Figure 4 where even the nuclear debris of the lymphocytes at nine hours after irradiation has practically been removed.

The changes in the thymus, though occurring an hour or so later than the changes of the spleen and lymph nodes, still show the early necrosis of the mature lymphocytes or thymocytes and the minimal changes within the cells of the reticuloendothelial system. These are illustrated in Figure 5 at two hours and Figure 6 at eleven hours.

This series of photographs demonstrates the typical changes which take place as a result of 650 rep gamma irradiation in our animals. Usually there is early and complete necrosis of lymphocytes and plasma cells. The cells which are affected during the time period in which antibodies are produced are those more undifferentiated cells of the phagocytic or reticuloendothelial system.

In the second series of experiments it was noted that transplants in the anterior chamber of irradiated recipients varied in their ability to produce

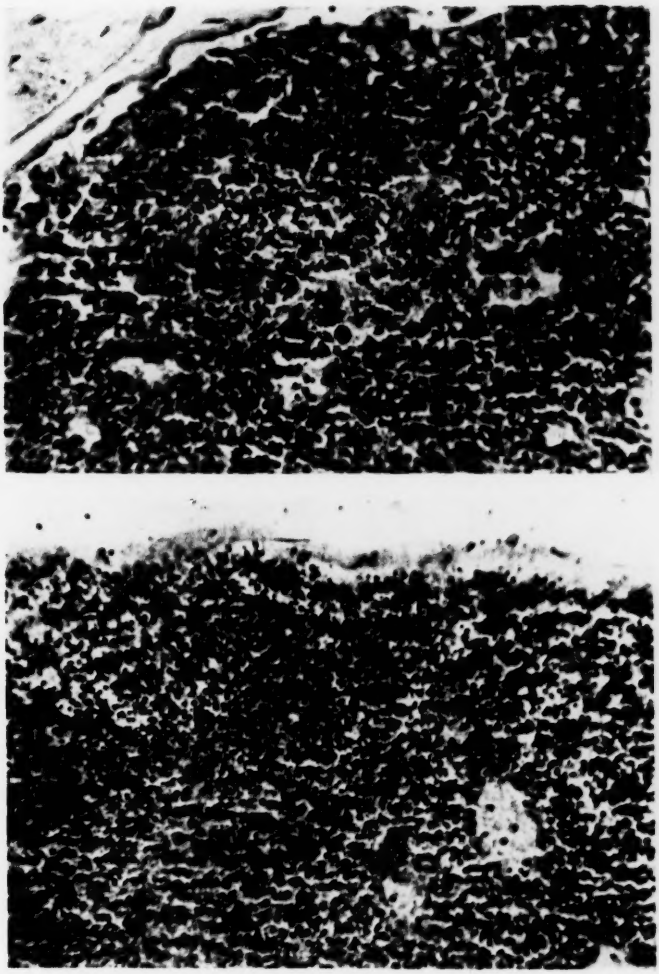

FIG. 1 (above). Axillary lymph node (×200, giemsa stain) one hour following 650 rep gamma irradiation. One focus of necrosis of lymphocytes with nuclear swelling of reticuloendothelial cells.

FIG. 2 (below). Axillary lymph node (×200, giemsa stain) seven hours following irradiation. Necrosis of mature lymphocytes with phagocytosis of nuclear debris and early nuclear swelling of reticuloendothelial cells.

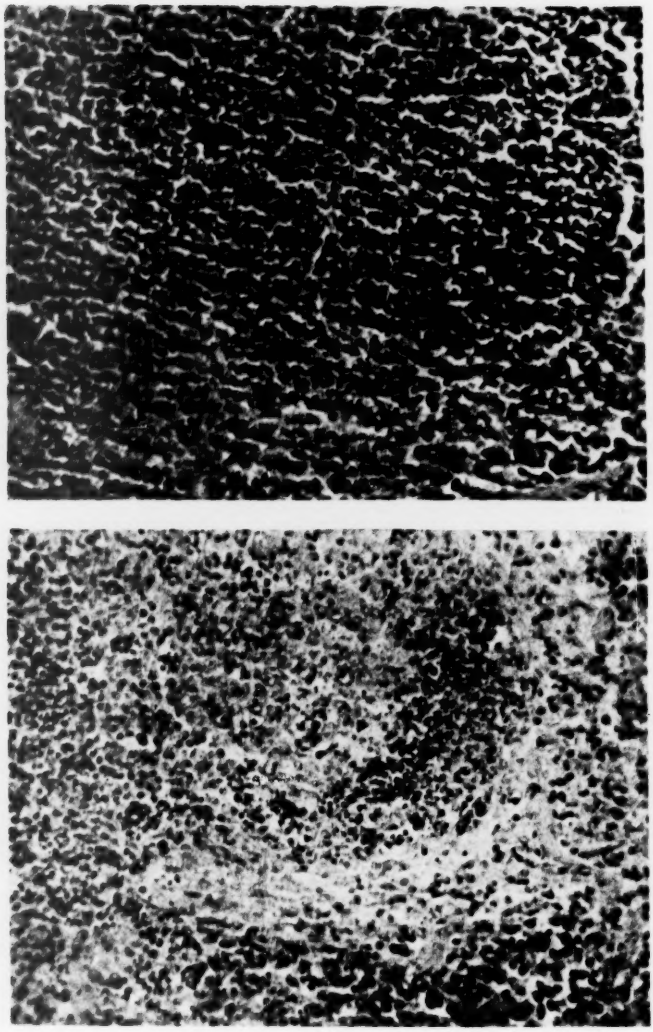

FIG. 3 (above). Spleen (×200, giemsa stain) three hours following irradiation. Note again karyorrhexis of lymphocytes and nuclear ballooning of stromal cells.

FIG. 4 (below). Spleen (×200, giemsa stain) nine hours following irradiation. Note absence of mature lymphocytes, phagocytosis of nuclear debris with partial removal, and extensive nuclear swelling of reticuloendothelial cells.

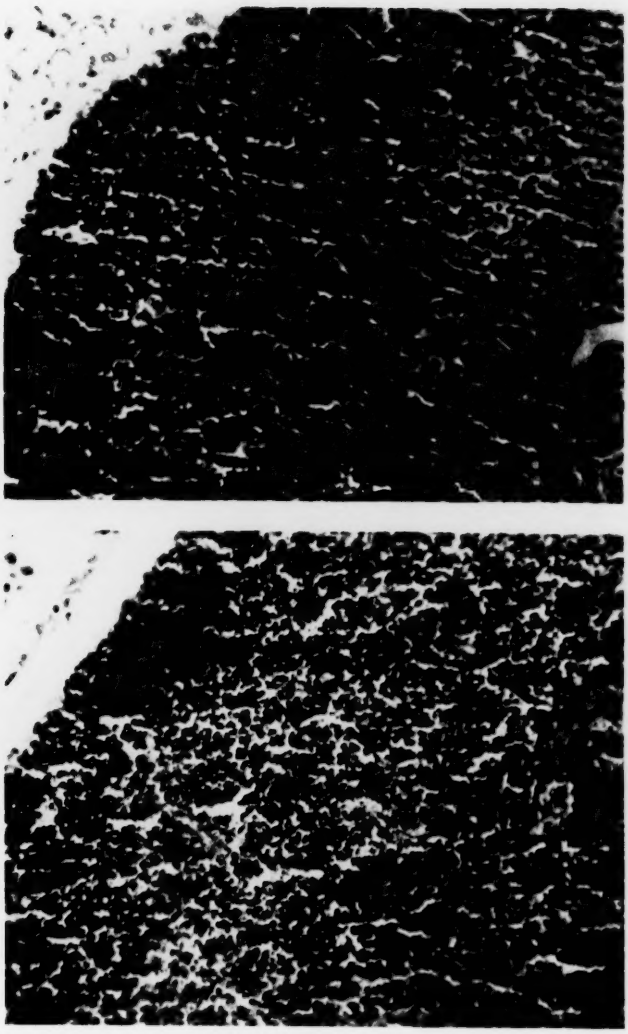

FIG. 5 (above). Thymus (x100, giemsa stain) two hours following irradiation. Note again necrosis of thymocytes and earlier nuclear swelling of stromal cells.

FIG. 6 (below). Thymus (x100, giemsa stain) eleven hours following irradiation. More extensive necrosis of cortical thymocytes.

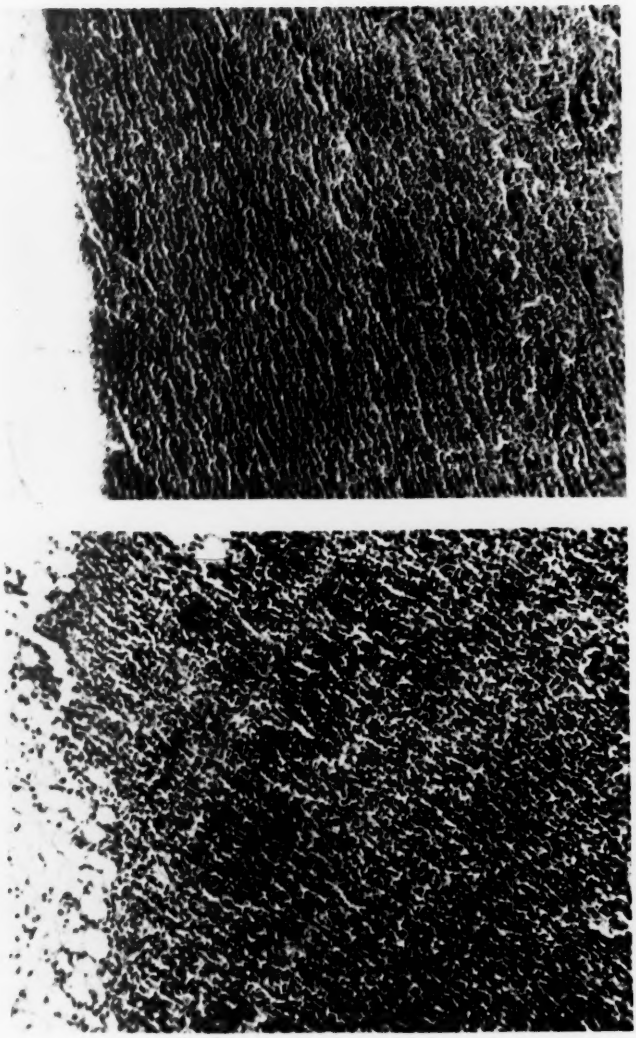

Fig. 7 (above). Normal thymus (x20, giemsa stain) of two-month-old mouse. Note regular scattering of thymocytes throughout cortex.

Fig. 8 (below). Normal thymus (x100, H&E stain) of 15-month-old mouse. Note prominence of stromal cells with only foci of thymocytes remaining and fat infiltration of cortex.

antitoxin depending upon the age of the donor mouse. Chart 2 illustrates the typical results of one experiment in which more antitoxin is produced by the older mice, regardless of whether the animals received a primary or secondary challenge. Transplants from older donors always produce as much, and usually more, antitoxin as do transplants from younger donors.

Figure 7 illustrates the normal cortical/medullary ratio in a young mouse (two months) in which the cortex is filled with mature lymphocytes or thymocytes. Figure 8 illustrates the aging changes of the thymus in a fifteen month old mouse. Here the lymphocytes are absent from the cortical region leaving only the more undifferentiated cells to be transplanted. This again illustrates that an absence of mature lymphocytes, or thymocytes, does not interfere with the ability to produce antitoxin.

DISCUSSION

The metabolic changes which influence the production of antibodies occur following the first six hours after irradiation. The mature lymphocytes and plasma cells that have been said to be the sites of antibody production have usually undergone complete necrosis within this six-hour period. The early changes reflecting the interference with antitoxin production are occurring in the cells of the phagocytic or reticuloendothelial system. This is also reflected in studies of age involution in the thymus where it is seen that the absence of mature cells does not interfere with antitoxin production. With these techniques we have not, however, been able to assess the rôle of lymphocytes and plasma cells in antibody production.

Terminology of these undifferentiated cells is confused. The stromal cells are classified under the reticuloendothelial system as defined by Aschoff[1] and later discussed by Marshall,[2] though their ability to accept the silver stain is altered somewhat by irradiation. The ability of these cells to phagocytize is established by the presence of nuclear debris within the cytoplasm of many of these cells. Evans and Scott[4] in their well-illustrated description of the two cell types within connective tissue illustrated that even the fibroblast will take up or phagocytize vital dyes under conditions of excess load.

Our concept of these cells which have the ability to produce antibody is that while they are members of the reticuloendothelial system, most of them have the ability to phagocytize under the proper stimulus. We believe that these represent a multipotential cell which may differentiate into a "transitional" cell and thence into a lymphocyte, plasma cell, or a phagocytic cell under the proper stimulus.

CONCLUSION

These experiments correlate (i) the morphological changes in lymphatic tissues following gamma irradiation with the alteration in ability of the animal to produce antitoxin and (ii) the morphology of aging of the thymus with its increasing ability to produce antitoxin in transplants. We believe we have demonstrated the ability of undifferentiated cells of the reticuloendothelial system to produce antibodies in the absence of mature lymphocytes or plasma cells.

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Section of Preventive Medicine and the Department of Internal Medicine, Yale University School of Medicine, and the Arthritis Clinic of the Grace-New Haven Community Hospital

THE HEMAGGLUTINATION TEST IN RHEUMATOID ARTHRITIS
I. USE OF RED CELL PATTERN AS ENDPOINT IN THE METHOD§

This is a description in some detail of the sensitized sheep cell hemagglutination test for rheumatoid arthritis as it is currently carried out in this Clinic. Sustained interest in the pathogenesis, natural history, and treatment of rheumatoid arthritis leads us to direct a significant portion of the investigative studies toward improvements in the serological method with fairly exact definition of its diagnostic and prognostic value.

A lengthy test and the vagaries of rheumatoid arthritis suggest that the description of the method and its evaluation be treated separately. The poorly defined clinical landmarks of rheumatoid arthritis do indicate the need for such a diagnostic test. It is when the limits of clinical discernment and serological accuracy are mutually established that both can be used to full advantage. This report is rather strictly confined to the actual procedure. Data at hand will supply the material for its clinical counterpart. Meanwhile, Table 1 and a recent publication[a] tell something of the performance of the hemagglutination test in rheumatoid arthritis.

MATERIALS AND METHOD

MATERIALS. 1. *Sheep red cells.* The sheep erythrocytes are obtained commercially as blood from a single sheep in an equal amount of citrate. The supply, which is received weekly, is refrigerated at 4° C. for a maximum of one month. The donor differs from week to week. Red cells are washed in saline at least three times. Packed volume under the clear supernatant is measured after centrifugation at 2,000 r.p.m. for 20 minutes. The preparation and resuspension of cells are arranged to coincide with the time of use. Twenty-five per cent red cells are obtained by direct dilution. The 1.0 per cent suspension is prepared by redilution of 10 per cent red cells.

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2. *Diluent.* Isotonic (0.89 per cent) saline is the diluent in all instances. Phosphate buffered solutions of a limited acid to alkaline range (pH 6.5, 7.0, and 7.8) are without value.

3. *Rabbit anti-sheep cell serum.* This glycerinated hemolysin (equal parts of glycerin and hemolysin) is also obtained from a commercial source. A 1:100 dilution is prepared as needed. Both the original supply and the stock dilution are stored at —10° C. The diluted hemolysin is discarded at the end of one week.

4. *Glassware.* Standard tubes (10 x 75 mm.) are used for the human serum throughout the method. Corks are included for storage.

METHOD. 1. *Absorption of heterophile (naturally occurring) agglutinins.* To 0.4 ml. of human serum (inactivated at 56° C. for 30 minutes) is added 0.8 ml. of a 25 per cent sheep cell suspension. The tube (10 x 75 mm. dimensions) is shaken and allowed to stand at room temperature for 15 minutes. It is shaken again and centrifuged at 2,000 r.p.m. for 10 minutes. Another 0.8 ml. of cell suspension is added to the supernatant and the tube shaken. The absorption process is thus repeated for an additional 15 minutes at room temperature followed by refrigeration at 4° C. overnight or for a minimum of one hour if the serum is to be used the same day. The suspension is shaken and centrifuged. The absorbed serum, now in a 1:4 dilution, is removed and used in the test or kept frozen at —10° C. The amount of easily removed serum is adequate for a repeat test, if needed.

A newer and less involved technique is the addition of 1.6 ml. of a 25 per cent sheep cell suspension to the 0.4 ml. of inactivated serum. The tube is shaken for a full minute and refrigerated. Absorption is started during the morning or early afternoon so that time allows a second and similar agitation. Centrifugation directly follows the overnight refrigeration.

Both methods are current. The latter is now routine, and the former is available when more than usual promptness is demanded. Information on the adequacy of both is included in the general discussion of the hemagglutination test which follows.

2. *Titration of hemolysin.* The dilution of hemolysin for the clinical test is established for each shipment of sheep cells. The desired amount is that which will agglutinate the cells at the established titer of the positive serum and clearly fail to cause agglutination in the normal serum. It is constant for each lot of cells but differs with the donor and even with the blood of the same sheep at another time. Two human serums are used. The one of known high titer is obtained from a patient with active rheumatoid arthritis, and the other is from an apparently healthy individual. The inactivated and absorbed control serums are from almost constant sources and are

stored at —10° C. For convenience, a sample of the high titer serum for current use is diluted to one-fourth of its titer and kept frozen.

Two sets of tubes are arranged in racks to contain five rows of four each. One set is used for each control serum. Three-tenths ml. of saline is added to the tubes. Serum in like quantity is placed in the first tube of each set. It is mixed and 0.3 ml. is carried by a pipette from tube to tube. The twofold serial dilutions of the normal serum are from 1 : 8 through 1 : 64 and those of the rheumatoid serum from 1 : 64 through 1 : 512. There are no control tubes since completeness of absorption was confirmed at an earlier time.

Five dilutions of the stock (1 : 100) hemolysin are usual. Concentrations are in the even numbers of 1 : 4,000 through 1 : 12,000. An additional tube (1 : 14,000) is occasionally indicated by recorded titrations with cells from a certain sheep. Experience allows ready interpolation to the nearest one thousand and a finer expression of hemolysin dilution is not considered practical.

Two milliliter amounts of 1.0 per cent red cells in saline are sensitized by addition to an equal aliquot of each dilution of hemolysin. The mixture is incubated in a water bath at 37.5° C. for 15 minutes and 0.3 ml. of even suspension is pipetted into each of the eight tubes. Thorough agitation of the racks is directly followed by overnight refrigeration. Incubation at 37.5° C. for one hour is no longer employed since the slight increase in titer is obtained at the expense of sharpness of pattern.

The desired pattern in both serums should be obtained without difficulty as the amount of hemolysin should not be so critical that some leeway does not exist. Sheep cells which are unsatisfactory are reserved for the absorption of serums, and re-titration includes the cells of another sheep. The results are regularly reproduced in the actual test where the two standard serums are again used.

3. *Clinical test.* A row of nine tubes is used for each sample of inactivated and absorbed serum. Saline in 0.3 ml. amounts is added to the last eight tubes. Three-tenths milliliters of patient's serum is placed in both the first and second tubes. It is mixed in the second tube and carried to produce twofold dilutions from 1 : 8 through 1 : 1,024. The first tube of a row contains serum in a 1 : 4 dilution for the detection of incomplete removal of heterophile antibodies. Dilutions of the two standard serums are limited to four.

Sheep cells are sensitized by adding an equal volume of 1.0 per cent red cells to hemolysin of a concentration set by the titration. This mixture of 0.5 per cent cells in diluted hemolysin is placed in a water bath at 37.5° C.

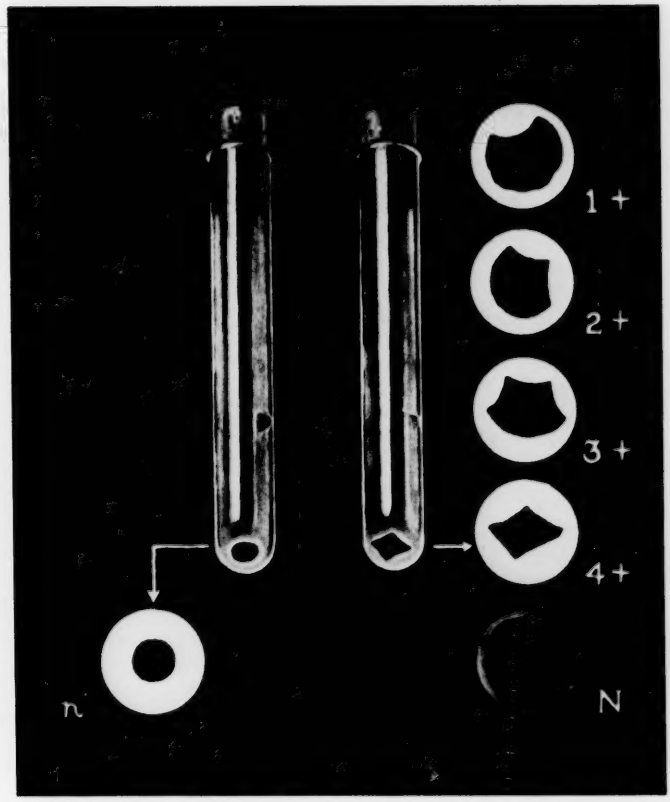

FIG. 1. Appearance of sheep red cells in the hemagglutination test for rheumatoid arthritis. The normal pattern of settled cells in nonrheumatoid (n) and usual background in rheumatoid (N) serums are shown. Clumping at periphery of patterns is recorded at 1 plus through 4 plus. Titer of serum is the highest dilution in which agglutination occurs. (1x and 4x magnification)

for 15 minutes, and then 0.3 ml. of the uniform suspension is added to the last eight tubes of each row. The first tubes (control) receive a like amount of unsensitized cells in a dilution of 0.5 per cent. The racks of tubes are well shaken and refrigerated overnight. The appearance of the settled red cells determines the titer and result. Checks on adequacy of absorption and correctness in the amount of hemolysin are supplied by the control tubes and dual standards. These with the serums under study complete a routine test.

4. *Results.* The appearance of the settled red cells determines the test result (Fig. 1). Titer is expressed in units as the reciprocal of serum dilution. A range of less than eight units through 1,024 units is usually more than adequate for testing. The positive and negative standard serums must give the expected reactions and adequate removal of heterophile agglutinins shown.

Patterns are observed effectively from below by holding the tilted tube (about 40 degrees) at eye level against good light. The typical "negative" pattern is compact and usually sharply defined. It best presents as a small red disc in the control tube when absorption is complete. In tubes containing sensitized cells the disc is usually larger and has a light center of varying size.

Reactions are graded one through four according to the extent of distortion at the periphery. The basic positive pattern is a thin film of cells which covers the bottom of the tube. Clumping of red cells to form a side is a one plus, and a square is a four plus. Two- and three-sided figures give the intermediate grades of two plus and three plus. These are the usual designs. Hexagons, pentagons, and triangles are assigned a four plus since there is complete loss of circular form. Clumping at the circumference of an N pattern is a one or two plus according to degree. The N or basic positive pattern and cupping with a less than linear distortion are recorded but are not yet used as the endpoint. Their inclusion will further increase titer and yet maintain specificity. In practice, a reaction of one plus or more places the titer, and a titer of eight units is a positive test.

DISCUSSION

The hemagglutination test for rheumatoid arthritis is described at some length from titration of the reagents to interpretation of results. This particular method has been in active use for over two years. Experience with the various modifications of the test extends over a longer but less continuous period of time. These modifications were originally used in the screening search for a single basic and fairly sensitive test. They are again

encountered by necessity in the evaluation of recent highly encouraging refinements[3,4,5] by others. Modifications occur naturally in the development of a test and lead to improvement. They can be too numerous, and a cumulative effect is felt when the new methods are imposed on diverse ones. A standard basic method permits ready evaluation of these promising refinements which deal principally with fractions of serum rather than whole serum.

The method as described detects the agglutination of sheep cells by settled pattern rather than by agitation. This is a more effective means since the titers of rheumatoid patients are unchanged or increased, while the serums of most nonrheumatoid individuals will fail to react within the visual range of the test. This is more than a matter of personal preference since the real range of the test becomes extended by the inclusion of the lower and now significant titers.

The total effects are not in conflict since *less* hemolysin is needed. It is the efforts to increase sensitivity by use of *more* hemolysin that have been ineffectual through an inordinate loss of specificity. This is apparent from the direct comparison of methods. It is expressed in a somewhat different way in the study of Alexander and de Forest.[1] Incidence of positive tests (64 units and more) was 75 per cent in rheumatoid patients. Selection of a titer of 1:16 (16 units) as the line of demarcation increased the per cent to 88 but positive tests appeared in individuals with osteoarthritis to the extent of 20 per cent.

This present method is suggested as a standard method. High selectivity is obtained without apparent alteration of the basic factors. Its nature suggests that more of the total mechanism is open to inspection. Both quality and consistency are important for accuracy in the appraisal of promising refinements.

The few other changes in the hemagglutination test are of a streamlining nature. The absorption procedure is the most effectual timesaver of all. Failure to remove adequately heterophile antibodies is so unusual that no effort is made to supply comparative figures. The following facts are immediately available. Absorption was incomplete in one of the serums from 121 recent patients (first method with refrigeration for two hours). This incidence of less than one per cent is known to be high. Removal was complete from all serums of 203 patients in a later group (second method). The treatment of exceptional serums is not yet uniform because of their infrequency and the efficacy of the usual means at hand. The process may be started anew with another 0.4 ml. sample of inactivated serum. Agitation and refrigeration is lengthened. A new lot of sheep cells is at times used. This means is usually preferred since the method remains constant. Time

and inadequacy of serum would favor the alternative of an addition of 0.8 ml. of cells to 0.6 ml. of the partially absorbed serum. The regular dilutions are maintained quite readily. There is loss of a safety factor since the concentration of serum in the control tube and first tube of the test is the same (1:8).

Although serum is used with continuing regularity in this laboratory, reference should be made to the use of plasma in the test. The following comparison was prompted in part by an early situation where only an oxalated sample of blood was available for the determination. The sources of plasma and serum for the parallel tests were 20 patients and 5 healthy adults. The patients were clinically distributed as: rheumatoid arthritis, 20; rheumatoid arthritis with psoriasis, 1; osteoarthritis, 7; and gout, 1. Those with rheumatoid arthritis included four whose tests had returned to nega-

TABLE 1. THE HEMAGGLUTINATION TEST IN RHEUMATOID ARTHRITIS AND IN NONRHEUMATOID ILLNESSES (200 ADULTS)

Clinical impression	*Number of patients*	*Hemagglutination test*	
		Positive	*Negative*
Rheumatoid arthritis	100	91	9
Other illnesses	100	2	98

tive and one with only negative tests on record. The titers of plasma and serum matched in 32 instances. Two individuals with rheumatoid arthritis had plasma titers of 512 units and serum titers of 256 units. There was no shift of result from negative to positive even with the five rheumatoid patients. For these and other reasons, the increase is without import. Neither absorption nor red cell pattern was affected. This is evidence that in usual laboratory practice plasma and serum can be used quite interchangeably as dictated by convenience or need.

Table 1 summarizes with minimal detail the results of the hemagglutination test in 200 patients. It is included since strict adherence to method may be unduly limiting in this discussion of a clinical test. Attention is called to the manner of selection of the patients. The primary source was the files of the Streptococcus laboratory which include many with rheumatic diseases. Clinical behavior of the test is assessed from the results in well-established illnesses, both rheumatoid and nonrheumatoid. The number in each group was limited to 100 adults. The rheumatoid patients have "textbook" rheumatoid arthritis and the result of the hemagglutination test tabulated is that of the initial determination. Similarly, the individuals in the control group have an accurately defined presenting illness without overt rheumatoid

arthritis. This should result in approximate information on sensitivity and selectivity with a somewhat high incidence of positive tests in both groups.

The data in Table 1 place the diagnostic effectiveness of the hemagglutination test at about 90 per cent (189 of 200 patients). Information obtained from other studies in this clinic indicates that the figures have a broad validity.[6] The failure of the method to support the clinical impression in the 11 patients lacks full explanation.

SUMMARY

The hemagglutination test for rheumatoid arthritis as described offers the high selectivity and sharp results which are requirements for a practical test. It should serve as a satisfactory standard method for new and special refinements.

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INFECTIOUS MONONUCLEOSIS AT THE YALE-NEW HAVEN MEDICAL CENTER 1946-1955†

This paper is presented in the form of a progress report with the primary objective of evaluating recent experiences with infectious mononucleosis observed at the Yale-New Haven Medical Center during the years from 1946 to 1955. Two preceding reports from this department, dealing with earlier experiences with this disease, have surveyed the 15-year period, 1923-1938[37] and the 8-year interval, 1936-1946.[38]

The current series totals 166 cases of infectious mononucleosis, diagnostically acceptable on the basis of clinical, hematological, and serological criteria. The basis of their original selection was that the clinical diagnosis of infectious mononucleosis had been made. This original list was carefully reviewed, case by case, and a number of questionable cases were eliminated. Frequently, the unabsorbed heterophile antibody titre was either negative or below a minimum titre of 1:80; although, by clinical and hematological standards, these cases *were acceptable* and included in the series of 166 cases as examples of infectious mononucleosis. These cases, which are 94 in number, have therefore been compared and analyzed throughout the paper with the group of 72 cases in which *all* criteria were satisfied. All of the cases have been admitted during the period, 1946 to 1955, to the Yale-New Haven Medical Center on either the medical or pediatric services and many were followed later in the out-patient departments of these respective services.

In the present review, no attempt will be made to cover all aspects of this important disease. Only recent literature will be reviewed and that in a relatively limited fashion. The emphasis in this paper, as in the previous ones, will be largely concerned with current theories relative to pathogenesis, etiology, and mode of spread, as well as new observations concerning the clinical picture and their meaning.

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REVIEW OF LITERATURE

Since 1946, numerous clinical and epidemiological developments with regard to this disease have received attention in the medical literature.

Epidemiology. The disease has been increasingly recognized in the past decade and its frequency is increasingly apparent. Currently, M. A. Rugg-Gunn[10] has reviewed a large series of cases of infectious mononucleosis in the British Royal Navy and finds it to rank second in frequency to rubella, if the common cold and influenza are excluded. However, there has been a significant lack of the occurrence of recognizable epidemics of bona fide examples of the disease. Although prevalent among hospital, university, and institutional population groups, infectious mononucleosis rarely has been observed among roommates and close associates. In their analysis of 47 cases in a New England college during the winter of 1948-49, Evans and Robinton[11] found only one instance of its occurrence in roommates, and observed that the disease tended to involve freshmen and sophomores more frequently than upperclassmen. At the United States Army Military Academy, Hoagland[15] also has noted the lack of prevalence of this disease in roommates.

Recent clinical experience. There has been an increased appreciation of the amount of diffuse organ involvement in infectious mononucleosis, particularly the liver. Liver biopsies have revealed a characteristic type of pathology consisting of mononuclear infiltration in periportal spaces and elsewhere, which can be differentiated from the pathology of infectious hepatitis. Evans[8] reported from this laboratory, positive cephalin flocculation tests in 95 per cent of cases without clinical jaundice. In a series of 15 cases without jaundice, Cohn and Lidman[3] correlated the severity of the disease with the amount of hepatic impairment and noted that the thymol turbidity and bromsulphalein excretion tests were of greatest clinical significance. In their series, Schultz and Hall[20] found evidence of abnormal bromsulphalein retention or abnormal flocculation tests in 92 per cent of 75 patients. In view of this fact that hepatic dysfunction is almost universal, liver function tests have been used by Watson *et al.*[26] in an epidemiological survey of subclinical cases.

Custer and Smith[5] state that interstitial pneumonitis occurring as a feature of infectious mononucleosis would be indistinguishable from that of atypical pneumonia, influenza, or rheumatic pneumonitis. In Evans and Graybiel's[12] series of four cases, electrocardiographic abnormalities were compatible with acute myocarditis.

Many investigators have called attention to varying degrees of encephalitis, meningitis, or both, occurring in this disease, and Custer and Smith[5]

have demonstrated these findings in three individuals. Two cases of their series presented the Guillian-Barré syndrome, and autopsy revealed only minor ganglion cell changes in the spinal cord, but there was the additional involvement of spinal nerve roots.

Thirteen cases of acute hemolytic anemia, complicating infectious mononucleosis, have been described in the literature, and were recently reviewed by Thurm and Bassen.[25] Recovery occurred in all cases of hemolytic anemia, but splenectomy was considered necessary in two cases of this series in view of the severity of the hemolytic process. The etiology of this complicating hemolytic anemia in infectious mononucleosis remains obscure, but Thurm and Bassen mention that it may be either the result of the liberation of hemolytic immune antibodies or a marked hypersplenic effect. Thrombocytopenic purpura, as a complication of infectious mononucleosis, and its regression during the period of convalescence have frequently been observed.

Experimental transmission. In contrast to the multiplicity of clinical observations in this disease, the results of experimental transmission of infectious mononucleosis have been less revealing. Repeated attempts to transmit the disease to primates, including chimpanzees, have been carried on in several laboratories including our own and have in almost every instance been unsuccessful. Evans[8] mentioned that prior to 1947 there had been 40 individual attempts at experimental transmission to man but only two were successful. Earlier work records the fact that in 1940, Sohier *et al.*[28] inoculated a human volunteer intramuscularly with 6 cc. of whole blood from a monkey which had been inoculated 16 days before with similar material from a patient with infectious mononucleosis. The volunteer later developed hematological and serological evidence of the disease, but had no clinical symptoms. Wising,[28] in 1942, used 250 cc. of heparinized whole blood from a patient in the acute phase of infectious mononucleosis, and injected this intravenously into a volunteer, who then developed an illness clinically suggestive of infectious mononucleosis, but with only a slight rise in sheep cell titre. In our own laboratory, Haven[6] in 1945-46 and Evans in 1946-47[8] repeatedly attempted experimental transmission in human volunteers by using pooled throat washings, serum and stool preparations from human cases of the disease. In the first experiments with 17 subjects, aged 19-25 years, 21 transmission attempts were made by using oral, intranasal, intramuscular, and intracutaneous inocula; however, in no instance did the human volunteer develop evidence of infectious mononucleosis and thus it was concluded that either the causative agent was labile and easily destroyed or that individual susceptibility was low in the volunteers employed.

Further experimental attempts by Evans in 1950,[10] in a group of 16 volunteers, produced suggestive diagnoses in seven subjects, but there was insufficient evidence to term these transmissions successful.

Recently, Hoagland[18] has suggested that on the basis of clinical experience, infectious mononucleosis may well be transmitted by direct and intimate oral contact, which allows for moderate salivary exchange, and that an incubation period of 33 to 49 days follows such exposure.

Differential diagnosis. In view of the increasing recognition of the protean clinical manifestations of infectious mononucleosis, the number of diseases for which it can be mistaken is expanding. It is not my purpose to present a complete listing of common diseases with which infectious mononucleosis may be confused, but among the most prevalent are: acute leukemia, infectious hepatitis, German measles, and acute appendicitis.

Acquired toxoplasmosis of the latent form may have generalized lymphadenopathy as a prominent feature, as well as a variable lymphocytosis. In this disease, a brief febrile period may occur, followed by lymphadenopathy persisting for several weeks and, in some instances, several months. Skipper, Beverley, and Beattie[22] have recently reported on two such cases of toxoplasmosis in young adults. In both instances, the Paul-Bunnell tests were negative. Siim[21] has commented that in some illnesses diagnosed clinically as infectious mononucleosis in which the Paul-Bunnell reaction is found to be negative, the serological reactions may well be positive for toxoplasmosis.

In 1942, Blake, Welt, and Craige[1] described a disease process characterized by transient fever, red-brown maculo-papular rash, lymphadenopathy, and leukopenia with lymphocytosis. The sheep cell titre was not elevated, and the presence of atypical lymphocytes was not uniform. They suggested, therefore, that this may be a variant form of infectious mononucleosis.

Cases of another differentiated disease, infectious lymphocytosis, have usually been described in the pediatric age group, although several adult illnesses have been reported. The majority of patients have exhibited signs of an upper respiratory infection, without further clinical symptomatology; however, abdominal pain and central nervous system signs may be associated findings. The significant characteristic is the leukocytosis, ranging, according to Riley,[18] from 22,500 to 120,000, with small mature lymphocytes varying from 62 to 97 per cent of the differential white cell count. This abnormal leukocytosis usually persists for two to five weeks, but lymphocytosis as long as seven months has been described. The heterophile antibody agglutination is negative in all cases.

Current literature contains numerous consecutive reports about the recent epidemic of 119 cases of an infectious disease,[3] which was initially thought to be infectious mononucleosis, at the Royal Free Hospital in London. This epidemic was of such magnitude that the hospital was forced to discontinue admitting patients for a limited time. The disease was characterized by malaise, sore throat, cervical adenopathy, headache, vertigo, and frequent signs of an encephalomyelitis. In no instance, however, were the typical lymphocytic abnormalities or elevated heterophile titre found to be present. This series of what might be called borderline illnesses indicates that there are a number of entities with which infectious mononucleosis may be readily confused, some well described, others very poorly delineated.

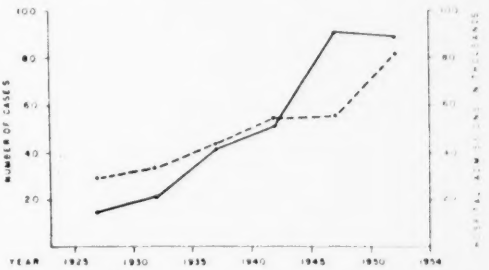

FIG. 1. Total cases of infectious mononucleosis admitted to the Yale-New Haven Medical Center during the five-year periods, 1925-1954, represented by the solid line. The broken line refers to total hospital admissions in thousands.

Present series of cases collected at the Yale-New Haven Medical Center, 1946-1955—Increase in frequency. A progressive increase in the number of annual hospital admissions has continued to be the experience at the Yale-New Haven Medical Center. Figure 1 illustrates this situation in graphic form with an incidence rising from a total of 15 cases hospitalized during the 5-year period, 1925-29, to 87 cases admitted between 1950-54. Although the number of hospitalized cases has increased steadily in the past 20 years, there has never been an apparent epidemic form of the disease either in the hospital, university, or urban population groups.

During the 1925-54 period illustrated in Figure 1, general hospital admissions to the Yale-New Haven Medical Center have also steadily increased. Their rise in the last four years (1950-1954) has been due in part to the consolidation of the New Haven Hospital and the Grace Hospital. The latter, however, accounts for a relatively small percentage (one per cent) of the total number of infectious mononucleosis patients admitted during that period.

It is my interpretation that this rising incidence is not necessarily due to an actual increase of the disease, but that two factors are important:

(1) the disease is being diagnosed more accurately and (2) the cases are being hospitalized with greater frequency.

Age. In this series of 166 cases, the maximum frequency occurred between the ages of 16 and 29 years, as illustrated in Figure 2. A similar age distribution pattern was established for the comparative group of 72 cases, chosen on the basis of more exacting diagnostic criteria. There was a significant number of childhood cases with 16.8 per cent present in children under ten years of age as noted in Table 1. This increase in juvenile cases is undoubtedly more apparent than real, but has been masked in the past by such mild symptoms that the disease passed unnoticed. Previous reports have emphasized the frequency of the disease in childhood, although this has not been the common experience of larger hospitals. It is probable that the concentration of cases between the ages of 20 and 30 in this particular series is due or at least weighted by the fact that the local medical students, internes, nurses, and the hospital attendant population of the Yale-New Haven Medical Center represent an appreciable part of potential patients.

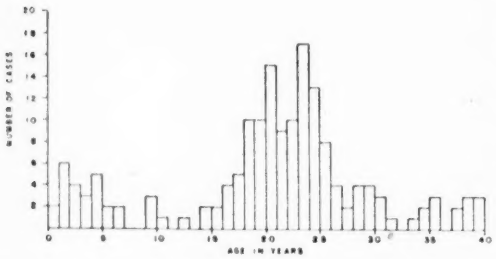

FIG. 2. Age distribution of 166 cases of infectious mononucleosis at the Yale-New Haven Medical Center, 1946-1955.

Season. In accord with previous experience, cases of infectious mononucleosis have not been concentrated in any particular season of the year. Actually, they have been distributed quite evenly throughout the year.

Race. Seven cases in this series were Negro patients representing 4.2 per cent of the total series. This is somewhat lower than the ratio of Negroes to whites in this general hospital population where the percentage of Negroes is approximately 10 per cent.

Occupational relationship. As noted in preceding paragraphs, the highest incidence of patients with infectious mononucleosis occurs between the ages of 16 and 29 years in this case analysis. This particular age distribution may be a prime determining factor in the occupational categorization of these patients, since approximately 50 per cent of patients have occurred among hospital, university, or institutional groups. The current series emphasizes the high, although sporadic, incidence of the disease, primarily

among medical students, nurses, internes, and secondarily among university students and institutional workers, which is in keeping with previous experiences. Numerous reports have also appeared in the literature concerning the frequency of the disease in military establishments (Wechsler *et al.*)[37] and training schools (Hoagland).[18]

Nevertheless, there must be other environmental factors involved in this disease which are over and above the mere exposure in hospitals and institutions, for otherwise one would be hard put to explain such findings as the significant number of cases in the pediatric age group.

TABLE I. AGE DISTRIBUTION BY DECADES OF 166 CASES OF INFECTIOUS MONONUCLEOSIS IN THE YALE-NEW HAVEN MEDICAL CENTER SERIES, 1946-1955

Age group	*Number of cases*	*Per cent in each age group*
0–10	28	16.8
11–20	48	28.9
21–30	75	45.2
31+	15	9.0

The occupational relationships of a *control* series of patients have been analyzed and the findings presented in Table 2. These controls were matched according to age, sex, and hospital year and all cases were admitted to the Yale-New Haven Medical Center during the period from 1946 to 1955. Five disease entities were used as a basis for the selection of controls, *i.e.*, (i) infectious hepatitis, (ii) rheumatic fever, (iii) scarlet fever, (iv) acute appendicitis, and (v) primary atypical pneumonia.

The most striking contrast between the current series of infectious mononucleosis cases and the above control group was the fact that hospital workers accounted for 32 per cent of the cases in this series, whereas they represent only 4 per cent of the control group. It is suggested that this may be due to the fact that the disease is more readily detected in a hospital population in view of a more careful clinical surveillance and additional laboratory tests. Also, hospital personnel would be admitted to the hospital for study perhaps more readily than members of the surrounding community.

In the control series, 24 per cent of cases represent hospital, university, and institutional workers, whereas over 46 per cent of the cases are from specific occupation groups, i.e., office workers, housewives, factory workers,

TABLE 2. OCCUPATIONS OF 176 CASES OF INFECTIOUS MONONUCLEOSIS COMPARED WITH A SERIES OF CONTROL CASES

Hospital attendants			*Institutional workers*			*Professional, technical, & kindred workers*			*Skilled & unskilled laborers & kindred workers*			*Other adult occupations*			*Children & young adults*		
	S	*C*		*S*	*C*		*S*	*C*		*S*	*C*		*S*	*C*		*S*	*C*
Med. Students	22	1	Univ. Students	22	32	Office Work.	6	8	Factory Work.	9	15	Housewife	11	30	Presch. Child.	22	25
Internes	5	1	Armed Forces	2	1	Salesmen	2	3	Laborers	4	3	No Occup.	3	2	Sch. Child.	18	24
Nurses	24	4	Camper	1		Veterinarian	1		Carpenter	1							
Technicians	2	1	Caretaker	1		Publisher	1		Plumber		1						
Attendant	1					Tel. Oper.	2	1	Farmer	1	1						
						Teachers		2	Mechanic		1						
						Dept. Store Work.		2	Cook		1						
						Musician	1		Housepainter		1						
						Dancer	1		Steward		1						
						Welfare Work.	1		Vendor		1						
						Police Off.		1	Window Clean.		1						
						Chauffeur	1		Repairmen		2						
									Maid	1							
Total	54	7	Total	26	33	Total	16	17	Total	16	28	Total	14	32	Total	40	49
Per Cent	32.5	4.2	Per Cent	15.6	19.9	Per Cent	9.6	10.2	Per Cent	9.6	16.9	Per Cent	8.4	19.3	Per Cent	24.1	29.5

S—Current series of cases of infectious mononucleosis.
C—Control series.

laborers, salesmen, etc. Among the professional and technical workers, there was no significant percentage difference between the infectious mononucleosis cases and the controls. Consequently, the designation of infectious mononucleosis as a so-called "white collar" disease was not borne out in this particular comparative case study.

TABLE 3. SYMPTOMS AND SIGNS DURING THREE CLINICAL STAGES OF INFECTIOUS MONONUCLEOSIS IN 166 CASES

First week 166 cases		*Second and third weeks 96 cases*		*Late and convalescent 15 cases*	
Fever	67%	GGE	53%	GGE	73%
Cervical aden.	58%	Cervical aden.	46%	Splenomegaly	47%
Sore throat	51%	Fever	44%	Hepatomegaly	13%
GGE	46%	Splenomegaly	29%	Malaise	13%
Malaise	42%	Anorexia	25%	Anorexia	7%
Headache	34%	Malaise	25%		
Red throat	27%	Hepatomegaly	24%		
Splenomegaly	25%	Sore throat	20%		
Chills	22%	Tonsillar exud.	15%		
Hepatomegaly	14%	Headache	14%		
Cough	13%	Red throat	11%		
Rhinitis	13%	Cough	8%		
Tonsillar exud.	13%	Nausea, vomiting	8%		
Anorexia	12%	Abdominal pain	6%		
Skin rash	8%	Skin rash	4%		
Photophobia	6%	Stomatitis	4%		
Abdominal pain	5%	Jaundice	3%		
Neck sore	5%	Diarrhea	2%		
Nausea, vomiting	5%	Photophobia	2%		
Diarrhea	3%	Swollen eyelids	2%		
Neurological Sx	2%	Neurological Sx	1%		
Jaundice	1%				
Stomatitis	1%				

CLINICAL PICTURE

Symptoms and signs. In view of the particularly insidious nature of this disease, clinical signs and symptoms will be analyzed in chronological stages, i.e., first week of the disease, second and third weeks, and late or convalescent period.

First week symptoms. The prodromal or incipient symptoms in this series (see Table 3) have been nonspecific in type but most important were: fever, tender cervical adenopathy, sore throat, and generalized glandular

enlargement. Malaise and headache were the next most prominent symptoms noted.

Actually, the symptomatology during this early period bore the same relationship in both the total case study of 166 individuals and the smaller comparative group of 72 individuals. The febrile period may become more exaggerated at the end of the first week, and in many cases may increase daily in a step-wise fashion. Frequent cases, however, experienced a high, sustained fever with the onset of the disease. In approximately 45 per cent of the cases in both series, generalized glandular enlargement was present by the end of the first week; 25 per cent of individuals had splenomegaly at this time. There was a one per cent incidence of definite clinical jaundice during the first week of illness in this series.

Neurological symptoms were present in a very small percentage of cases, and the most severe complication in this series was that of a transient, left facial paresis which the patient developed on the fourth day of illness. Lumbar punctures were performed in 20 cases with the subjective symptom of headache but without objective neurological findings and 8 of these individuals (40 per cent) had elevated CSF protein determinations, ranging from 45 mgm.% to 119 mgm.%. A pleocytosis was present in the CSF of 4 cases (20 per cent).

Second and third weeks or mid-stage symptoms. Approximately 58 per cent of the cases in this series experienced persistent symptoms during the second and third weeks. It is in the second week of the disease that the full clinical picture becomes manifest. Generalized glandular enlargement, cervical adenopathy, and fever were the three most prevalent symptoms, and splenomegaly and hepatomegaly were present in about 30 and 25 per cent of the cases, respectively. Pharyngeal complaints were frequent, with sore throat being more commonly noted than either the objective findings of tonsillar exudate or pharyngitis.

Clinical jaundice was observed in only 3 per cent of cases during the second week of illness. In 47 nonjaundiced patients, liver function tests were performed during the second- and third-week period and were abnormal in 85 per cent of these individuals.

Late or convalescent symptoms. Fifteen cases (9 per cent) in this series had either objective or subjective symptoms beyond three weeks. Generalized glandular enlargement continued in 73 per cent; splenomegaly persisted in 47 per cent of the individuals. Symptoms of malaise and anorexia persisted in some instances for several weeks, and one case experienced generalized adenopathy and low-grade fever for a period of 12 months.

There were no deaths in this series, and the most severe complication was that of an associated hemolytic anemia, which was present in two cases, both of which responded well to cortisone therapy. In one case, electrocardiographic abnormalities consisting of T wave depressions in Leads I, II, V4-6 were present.

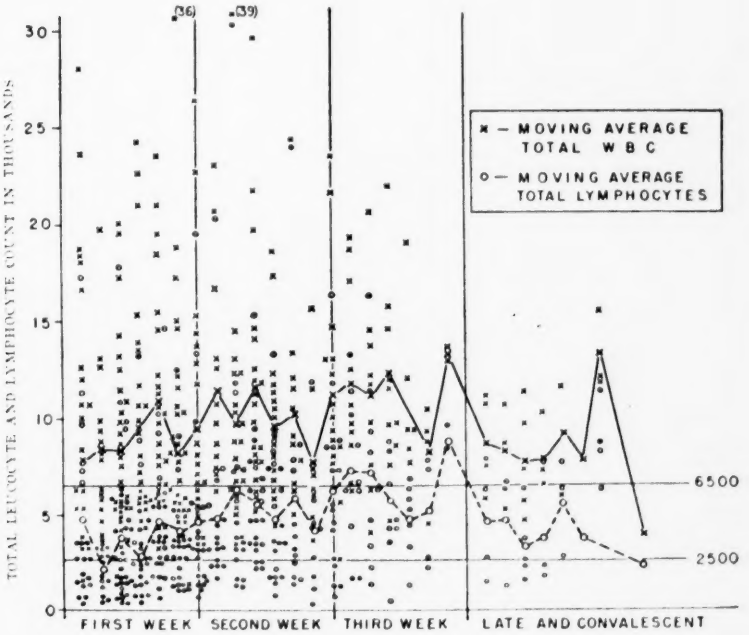

FIG. 3. Leukocyte and lymphocyte determinations in 166 cases of infectious mononucleosis.

Blood picture. The patterns of total white blood count and differential count are summarized in Figure 3. These data are based on an analysis of 347 blood counts in 166 patients.

In many ways, this complication of data yields disappointing results in that the aggregate counts during the first week of the disease did not depict a trend observed in many individual patients, i.e., an initial leucopenia, followed by a moderate leucocytosis developing during the second week of the disease, and reaching a peak at approximately the 15th day of the disease. Many of the present series had a leucocytosis during the first week of the

disease which persisted throughout the second and third weeks of the illness. Lymphocytosis increased at the end of the first week of the disease and persisted throughout the second and third weeks of illness. The average degree of lymphocytosis during this period was between 50 and 70 per cent.

The two cases of this series which were complicated by hemolytic anemia developed this complication during the second week of the disease. Hematocrit levels dropped from 47-32 and 42-32, respectively, and were associated with elevated reticulocyte counts. In both instances, the Coombs test was negative.

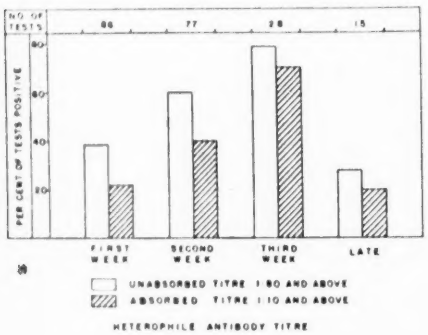

FIG. 4. Heterophile antibody determinations in 166 cases of infectious mononucleosis.

There was no laboratory evidence of associated thrombocytopenia in this series of cases.

The heterophile antibody titre. Recent work by Zarafonetis, *et al.*[29, 30] has demonstrated that in many instances false positive heterophile agglutination results may be due to the phenomenon of cold agglutination and they have advocated that cold agglutinins for sheep red cells be removed by absorption with guinea pig kidney or dispelled by warming to 37° C. It is suggested that cold agglutinins may have been responsible for the confusing results earlier with the Paul-Bunnell test.

The actual tests used for clinical diagnosis in this study have been of two types: (i) the Evans test[7] for prompt diagnosis, which is more or less a screening procedure, (ii) the other representing a modification of methods used by Davidsohn[8] and Stuart *et al.*[24] In all cases, adsorption tests were performed with guinea pig kidney. This method has been described in a bulletin[4] issued by the Bureau of Laboratories, Connecticut State Department of Health.

In the present series, we have considered significant only an unadsorbed heterophile titre of 1 : 80 or higher, but recognize any completely adsorbed titre with guinea pig kidney of 1 : 10 or greater* as being serologically valid in infectious mononucleosis. The degree to which positive sheep cell

* In this series, 23 per cent of cases had a completely adsorbed titre with guinea pig kidney of 1 : 10 or 1 : 20; 77 per cent were higher.

agglutinins occurred during weekly intervals of the disease appears in the diagram, Figure 4.

The data are based on the results of 200 serological tests. It will be seen that during the first week of illness, only 38 per cent of the cases had significantly positive titres, but that this increases to 60 per cent during the second week, and reaches a peak of 79 per cent positive in the third week of the disease. A precipitous decline in heterophile antibody titre then follows in the fourth and subsequent weeks.

It is apparent from these data that the diagnosis should not be made on the basis of one test alone, and that sequential tests should be used. In view of the sharp decline in titre after the third week, it appears, as has been suspected in the past, that we are dealing in this disease with a transient and short-lived antibody.

Comment and summary. This analytical report of the hospital records of 166 cases of infectious mononucleosis, admitted to the Yale-New Haven Medical Center during the period, 1940-1955, is the third of such progress reports from this particular institution. Although it was felt that each of the above 166 cases was clinically diagnostic of infectious mononucleosis, in many cases the heterophile antibody titres were either negative or not significantly elevated. Thus, the results of this larger number of cases were compared with a group of 72 cases in which serological criteria were diagnostic and found to be essentially the same. Consequently, analytical results were considered to be statistically valid in all of the original 166 cases.

The frequency of diagnosis of infectious mononucleosis was noted to be steadily increasing, and it is the author's opinion that this indicates an increasing degree of familiarity with the disease and its recognition on the part of physicians in general. No epidemic form of the disease has been apparent in this population group. The age distribution of this series revealed maximum incidence to occur between the ages of 16 and 29 years, and there was an increased number of cases diagnosed in the pediatric age group. As previously noted, no seasonal variations could be established.

The occupational relationships of these cases were reviewed and approximately 50 per cent of the patients came from hospital, university, or institutional groups. A control series of patients, matched according to year, sex, and age, was examined and the marked increase of infectious mononucleosis in the hospital population was substantiated by comparison. However, among nonmedical professional and technical workers, there was no apparent difference between the study and control groups; thus, the designation of infectious mononucleosis as a so-called "white collar disease" could not be substantiated in this particular analysis.

Clinical features of the disease were reviewed and certain complications present in this series were included. An analysis of the blood picture was presented and an early leukopenia followed by a significant rise of the lymphoid elements beginning at the end of the first week of the disease was observed in many cases.

The heterophile antibody titre was found to be significantly positive in 38 and 60 per cent of tests during the first- and second-week periods, with a maximum of 79 per cent of tests diagnostically positive during the third week. The value of sequential determinations of heterophile antibody titre for a definitive diagnosis was noted.

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A TECHNIQUE OF CROSS-CIRCULATION IN THE RAT WHICH PERMITS ACCURATE CONTROL OF BLOOD VOLUME TRANSFERS‡§

While the technique of cross-circulation has been used extensively in physiology, it has usually been employed with large animals. The present paper describes a method of cross-circulation that has been successfully adapted to the rat.

The use of this species offers certain advantages: (a) animals are uniform in structure as a result of continued inbreeding, (b) blood types are similar and consequently difficulties due to incompatibility are not a problem, (c) marked resistance to infection is present, (d) certain organs can be removed comparatively easily, and (e) since the laboratory rat has been used extensively in biological investigations, a great deal of standardized information is readily available for use as control data.

PREPARATION OF ANIMALS

Rats weighing approximately 200 grams have been used. They are anesthetized with Nembutal (5 mg. per 100 gm. body weight given intraperitoneally) and are prepared as follows:

A transverse skin incision is made just below the inguinal ligament; the skin is dissected free of fascia and retracted, so that an adequate exposure of the femoral artery, vein, and nerve is made. The blood vessels and nerve are dissected free of adhering connective tissue and the femoral nerve is cut in order to diminish the number of impulses relayed during the cannulation and cross-circulation procedures. A 2 per cent solution of Papaverine[a] is then applied locally to the blood vessels to relieve the vasospasm that occurs

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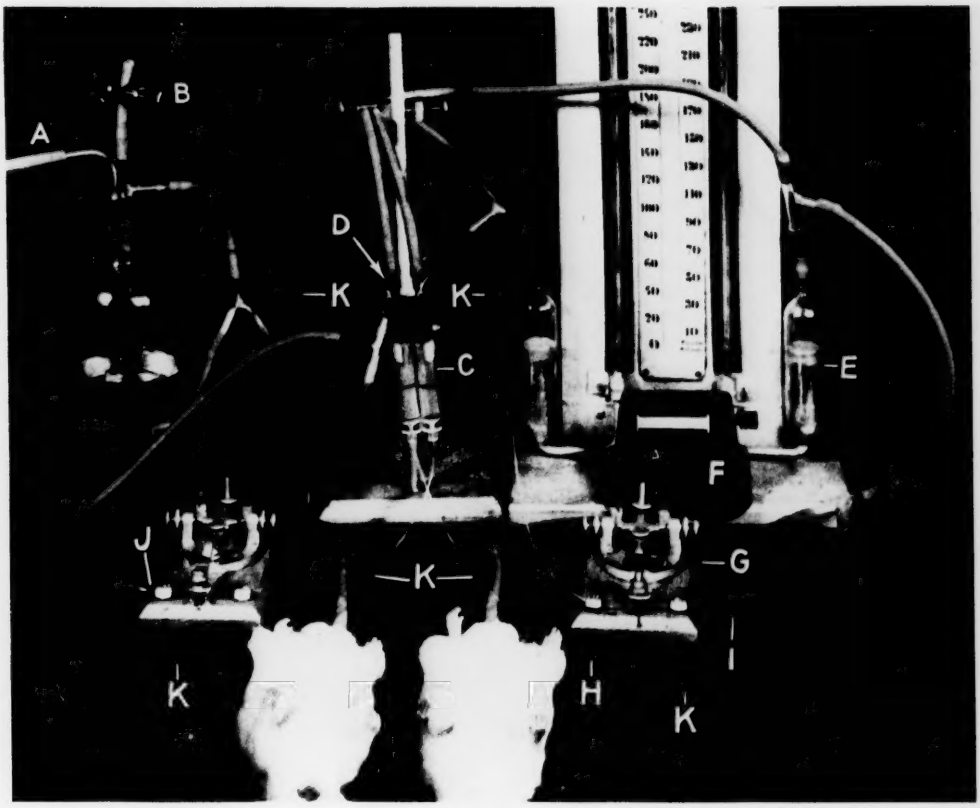

FIG. 1. Photograph of the cross-circulation apparatus.

A. Air from main line bubbling through water.
B. Fine adjustment screw for delivery of desired air pressure.
C. Lucite chambers graduated at 1 ml. mark.
D. Hypodermic needles, passing through rubber stoppers, to deliver air into reservoir.
E. Mercury manometer to measure applied air pressure.
F. Counter used to record volume transferred.
G. Telegraph key to regulate cross-circulation of each animal independently.
H. Polyethylene cannula (PE 50 — o.d. = .038") joined to polyethylene tubing (PE 90 — o.d. = .050") for arterial outflow.
I. Compressible tygon tubing (o.d. = .065") passing through key joining arterial outflow tubing with arterial inflow into reservoir.
J. Rubber tubing joined (by means of hypodermic needle) to compressible tygon tubing (o.d. = .065") for air release cutoff.
K. Polyethylene tubing (PE 90 — o.d. = .050").

(o.d. = outside diameter)

as a result of manipulation of the femoral vessels. Thread ligatures are placed under the vessels to be cannulated, and the entire operative area is kept moist with warm saline. Polyethylene tubing (size P.E. 50), filled with a solution of heparin (10 mg./ml.) and occluded at one end, is used to cannulate both the femoral artery and the vein. While the central and peripheral ends of the blood vessels are occluded by means of the ligatures, a transverse incision is made in the blood vessel wall and, with the aid of a fine dental pick used as a guide, the cannula is inserted into the blood vessel and tied securely in place. The peripheral ends of the blood vessels are then tied off and the ligatures removed from the central ends. After cannulation of the Donor and Recipient animals has been completed, both receive intravenous injections of 1 mg. of heparin in a volume of 0.1 ml. and are connected to the cross-circulation apparatus.

CROSS-CIRCULATION APPARATUS

An apparatus was designed (Figs. 1 and 2) constructed of plastic nonwettable surfaces, which could accurately measure and transfuse specific, relatively small, volumes of blood, of the order of 5-100 ml., from one animal to the other. For this purpose, two lucite cylindrical chambers of approximately 2 ml. capacity, and graduated at the 1 ml. mark, are used as blood reservoirs for each animal. The animal's vascular system is connected to the reservoir by means of polyethylene tubing (size P.E. 90) which could be joined to the cannulae without danger of leakage. The blood is always in contact with plastic surfaces and flows freely without clotting. The cannulae from the femoral arteries enter the head of the cylinders while the venous return to the femoral veins is attached at the base, and of course crossed over, so that the arterial blood of one animal is discharged into the femoral vein of the other. Since the chief difficulty in cross-circulation experiments in small animals is an inadequate rate of venous return through the fine polyethylene tubing employed, provision is made to apply air pressure to each cylinder. This not only rapidly empties the contents of the cylinders into the veins, but at the same time is so arranged that the arterial cannulae are occluded throughout this period. A simple telegraph key in conjunction with a mercury manometer is used to control both the magnitude and duration of the applied air pressure, so that the operator can adequately control the volume cross-transfused from one animal to the other. Thus, when the reservoir is filled to the 1 ml. mark, the operator presses the telegraph key which simultaneously occludes the arterial inflow, occludes the air pressure outflow from the reservoir, and opens the air pressure inlet into the reservoir, thereby building up a pre-determined magnitude of air pressure

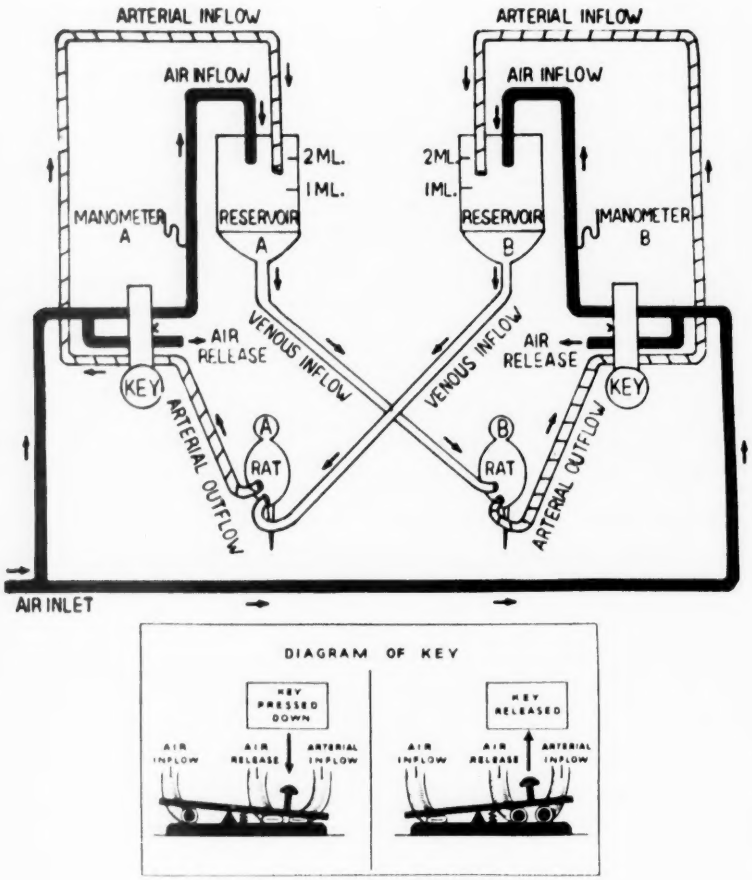

FIG. 2. Diagram of the cross-circulation apparatus.

(approximately 100-150 mm. Hg). The blood is forced out of the reservoir and into the vein at a rate dependent upon the air pressure applied. Upon release of the telegraph key, the pressure in the reservoir is diminished almost instantaneously and the outflow of blood from the reservoir ceases. In this way, 1 ml. of arterial blood can be collected from the Donor rat A in one cylinder and discharged into Recipient rat B. Each emptying of each reservoir is recorded. Since the filling and emptying of the cylinders are independent of each other there is no possibility of bleeding one rat into

another and the rate of exchange of blood can be kept equal. Blood volumes up to 100 ml. have been cross-circulated; the average rate of transfer is approximately 1 ml. per minute. At the end of the cross-circulation of a specific volume of blood, the blood vessels are tied off and the cannulae removed. The animals readily recover from this procedure.

DONOR AND RECIPIENT BLOOD MIXING DURING CROSS-CIRCULATION

Calculated equilibrium curve

When using a cross-circulation technique in biological research, it is often necessary to know the number of transfers required for the circulations of the cross-circulated partners to attain equilibrium. An equation may be derived to calculate the degree of blood mixing at any time during cross-transfusion.

$$C_{Bn} = K_3 \left(\frac{K_4^n - 1}{K_4 - 1} \right) + K_4^n C_{B0}$$

where:

C_B = conc. in partner B at any time.
C_A = conc. in partner A at any time.
y = ml. transferred at each interchange.
n = number of transfers.
V_A = volume of blood in partner A.
$V_a = V_{A0} + y$.
V_B = volume of blood in partner B.
$V_b = V_{B0} + y$.
$K = C_{B0}V_{B0}$.

$$K_1 = \frac{K}{V_{A0}}.$$

$$K_2 = \frac{V_{B0}}{V_{A0}}.$$

$$K_3 = \frac{K_1 y}{V_B + K_2 y}.$$

$$K_4 = \frac{V_b}{V_B + K_2 y}.$$

Calculation of the degree of mixing by means of this equation requires specific information such as the initial blood volumes of the two animals, the volume transferred during each interchange, and the initial concentration of the substance to be assayed in both A and B. Furthermore, it must be assumed that the material being assayed is not added to or lost from the circulations at an appreciable rate.

Using this equation, the per cent mixing was calculated for animals of different initial blood volumes, i.e., 10 ml., 15 ml., and 20 ml., using transfer volumes of 1 ml. per exchange. The results of these calculations are shown in Figure 3. It can be seen that animals with smaller blood volumes reach equilibrium with one another sooner than do animals with larger blood volumes.

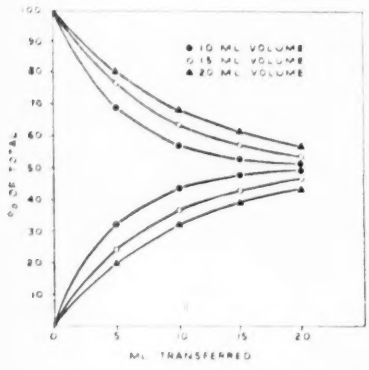

FIG. 3. Equilibrium curves obtained by means of calculation from equation (see text).

Equilibrium curve experimentally determined by means of Fe^{59}

Experimental verification of the calculated equilibrium curve was made by the use of radioactive isotopes. Fe^{59} was administered to Donor rats two weeks prior to cross-circulation with normal Recipient animals whose red cells were not labelled. During this two-week interval, the radioactive isotope was incorporated into the Donor's red blood cells, which now served as the indicator for the degree of blood mixing, since the red blood corpuscle is not appreciably lost from the circulation during a short-term experiment. Sprague-Dawley rats of 200-300-gram body weight were used in this study. The radioactivity of 0.2 ml. blood samples, which were withdrawn at specific intervals during the cross-circulation, was measured by means of a scintillation counter. The averages of the results of four such experiments are presented in Figure 4. The results indicate that equilibrium (at least 95% mixing) between the two circulations is attained after twenty 1 ml. transfers.

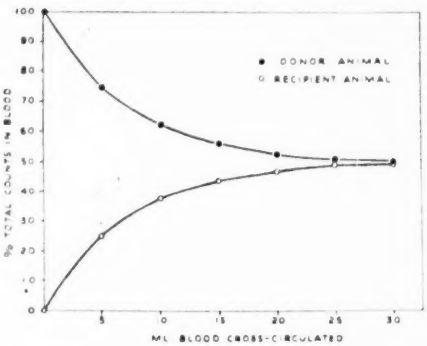

FIG. 4. Blood mixing curves between Donor and Recipient using radioactive Fe^{59}.

COMMENT

The method described in this paper has been successfully employed in the study of certain problems in endocrinology[1] and hematology.[2] It is anticipated that this technique of cross-circulation in the rat, which permits accurate control of blood volume transfers, will find numerous applications in biological research.

SUMMARY

The technique of cross-circulation has been adapted for use on small animals such as the laboratory rat. The technique and apparatus are described in detail. The femoral artery and vein are cannulated and specific volumes of blood are cross-transfused from one animal to another. The apparatus is so designed that the blood volumes of the two animals are kept in balance. The rate of transfer is approximately 1 ml. per minute, and volumes as large as 100 ml. have been exchanged without detriment to the partners. Using labelled red cells, it has been found that twenty 1 ml. transfers result in equilibrium between the two circulations.

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ESTIMATION OF BLOOD ACTH BY MEANS OF A CROSS-CIRCULATION TECHNIQUE‡§

Since no direct chemical method is at present available for the quantitation of ACTH secretion, indirect indices have, of necessity, been employed. Although many physiological and biochemical changes are associated with an increased activity of the pituitary-adrenal system, only a few of these are applicable as a method for the detection of such activity.

Up to the present time, the detection of the release of ACTH from the anterior pituitary has been carried out by one of two methods. The first is to observe the change in one of the indices of ACTH secretion in the animal to which an experimental procedure under study has been applied. For example, the decline in the adrenal ascorbic acid has been extensively employed as an index of ACTH secretion. However, in the intact animal, this only indicates that an increased discharge of ACTH has taken place, but gives no information as to the duration of the increased secretion of ACTH. This is because the increased activity of the adrenal cortex continues for a long time after a brief stimulus with ACTH,[18] despite the fact that ACTH is inactivated with extreme rapidity.[16]

The second, more quantitative, method for the determination of ACTH in blood, used by Sydnor and Sayers,[14] Gemzell,[5] Cooke,[4] Bornstein,[1] Parrott,[6, 9, 10] and others, is to pool the blood of a large number of rats subjected to the same procedure. After suitable extraction and chemical manipulation, the concentrated material is injected into hypophysectomized rats, and the ACTH estimated by means of the decline in adrenal ascorbic acid according to the standard method of bioassay developed by Sayers, *et al.*[13] While this method can be employed to determine the blood ACTH at

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§ The material presented herein was taken from a dissertation submitted in 1955 to the Graduate School of Yale University by A. Brodish in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology.

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different times following the application of a stimulus, it obviously offers opportunities for loss of activity through chemical changes to which the blood is subjected. That this occurs is suggested by the reports of Morruzzi and coworkers[7] who claim to have demonstrated the existence of two types of ACTH in blood: (a) an "active ACTH," found mainly in normal subjects, which can be detected directly in the blood but which cannot be extracted and concentrated on oxycellulose, and (b) an "activable ACTH," found primarily in subjects with hypocorticism, which cannot be detected in blood directly, but can be estimated after activation and concentration with oxycellulose. Parrott,[10] comparing the acid-acetone extraction with the oxycellulose extraction of ACTH, found good recoveries when these methods were applied separately, but a significant loss of ACTH activity occurred if both extraction procedures were employed on the same sample of plasma. Similarly, Gemzell, *et al.*,[6] using the acid-acetone extraction procedure, were unable to detect ACTH activity in 448 ml. of plasma from intact rats, whereas Sydnor and Sayers,[14] using the oxycellulose technique, were able to demonstrate activity in 12 ml. of blood from intact rats.

In an attempt to avoid these difficulties, a cross circulation technique has been developed whereby the blood level of ACTH, in its natural state in the circulation, may be measured. The operative technique and apparatus used has been described in the preceding paper.[2] The principle of the method is as follows:

If a normal rat is united with a hypophysectomized animal, the amount of ACTH in the blood of the intact rat can be estimated by the change produced in the adrenal ascorbic acid of the hypophysectomized partner. One adrenal is removed from the hypophysectomized Recipient before the cross-circulation and the second gland is removed after a known quantity of blood has been exchanged between the animals.

EXPERIMENTAL METHODS

Male rats of the Sprague Dawley strain, weighing 200-300 grams, were used in these experiments. The "test" animal, (called the Donor), whose ACTH output was to be measured, was kept in a constant temperature room for at least one week prior to the experiment, and fed a diet of Purina chow and water, ad libitum. At the time of the experiment, the animal was anesthetized with sodium pentobarbital (Nembutal 60 mg./ml.), 5 mg./100 gm. body weight, given intraperitoneally. The Recipient "assay" animal was a rat that had been hypophysectomized 36-84 hours previously.

The operative cannulation procedure and apparatus for cross-circulation are described in detail in the preceding paper.[2]

By means of the dorsal approach, one adrenal was removed from the hypophysectomized Recipient prior to the cross-circulation. This adrenal served as the control gland and was dissected free of adhering tissue, weighed on a Roller-Smith torsion

balance, and placed in 6% Trichloracetic acid for subsequent analysis of ascorbic acid concentration according to the method of Roe and Kuether.[11]

Prior to the beginning of cross-circulation, both Donor and Recipient received intravenous injections of 1 mg. of heparin in a volume of 0.1 ml. At the end of the cross-transfusion of a specific volume of blood, the femoral blood vessels were tied off and the polyethylene cannulae were removed. Approximately 15-30 minutes after the end of the cross-circulation, the second or experimental adrenal gland of the Recipient was removed, cleaned, weighed, and prepared for subsequent ascorbic acid analysis. At the termination of the experiment, completeness of hypophysectomy was determined by a careful examination of the sella turcica. The results obtained from those experiments in which the Recipient was incompletely hypophysectomized were discarded.

TABLE 1. REDUCTION OF ASCORBIC ACID IN THE ADRENALS OF HYPOPHYSECTOMIZED RATS BY CROSS-CIRCULATION OF VARYING AMOUNTS OF BLOOD OF NORMAL RATS

No. of expts.	*Vol. blood X-circ. ml.*	*Duration of X-circ. min.*	*% Change in adrenal ascorbic acid of hypox. ± S.E.*	*P*
17	0	...	+ 2.4 ± 0.73	
10	5	6.3	+ 1.7 ± 1.87	> 0.5
10	10	17.5	— 10.2 ± 1.87	< 0.01
24	20	24.8	— 17.5 ± 1.70	< 0.01
10	40	52.8	— 28.2 ± 3.76	0.01
10	60	63.9	— 30.7 ± 2.39	> 0.5
10	80	79.0	— 35.3 ± 3.47	0.2
6	100	96.7	— 35.5 ± 4.46	> 0.5

RESULTS

Using normal Donors and hypophysectomized Recipients, cross-circulation was established and varying volumes of blood, ranging from 5 ml. to 100 ml., were transferred from one animal to the other. The results of these experiments are presented in Table 1. They indicate that a relationship exists between the volume of blood transferred and the percentage change in the adrenal ascorbic acid of the Recipient. Significant differences were obtained between volume transfers of 5 ml., 10 ml., 20 ml., and 40 ml. However, a blood volume transfer of more than 40 ml. did not further increase the adrenal response as judged by the ascorbic acid depletion method. It is interesting to note that 5 ml. of cross-circulated blood exerts no demonstrable effect on the adrenal of the Recipient, whereas an additional 5 ml. produces a marked effect.

By this method, a linear relationship has been found between the logarithm of the volume of blood transferred from the intact rat and the fall

in adrenal ascorbic acid of the hypophysectomized animal (Fig. 1). Examination of this linear correlation indicates that under the conditions of these experiments the Recipient does not begin to respond until more than 5 but less than 10 ml. of normal Donor's blood have been transfused; in other words, the method is insensitive to that amount of ACTH which is normally present in 5 ml. of the blood of normal cannulated Donors.

In experiments utilizing the cross-circulation technique, one of the important variables which must be evaluated is the duration of the cross-circulation. This factor is important in determining whether the percentage change in the adrenal ascorbic acid of the hypophysectomized Recipient is in response to the total amount of ACTH presented to the adrenal or whether the gland is responding to the duration of the increased concentration of ACTH in the blood. If the latter is true, then the time of cross-circulation should be a significant factor in these experiments. Table 1 indicates that as the volume of blood cross-transfused is increased, the duration of the cross-circulation is increased concomitantly. Therefore, the increased response of the adrenal could conceivably be due to the prolonged duration of transfer of a given quantity of ACTH, which would thereby expose the adrenal to an increased concentration of the hormone for a longer period of time.

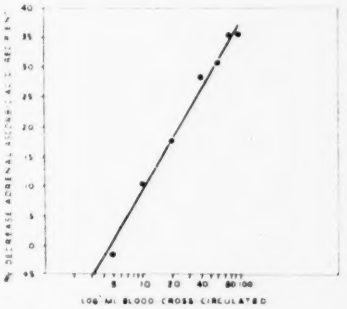

FIG. 1. Dose-response curve. The effect of volume of blood cross-circulated from normal Donors on percentage change in adrenal ascorbic acid of hypophysectomized Recipients.

However, if the 24 experiments (see Table 1) in which a blood volume of 20 ml. was transferred are subdivided according to the length of time necessary to cross-circulate this volume into the Recipient (i.e., same amount of ACTH), then a change in the adrenal response will become evident if the duration of cross-circulation is a factor. Table 2 shows that the variations in the time necessary to transfer a given quantity of ACTH exert no appreciable effect on the adrenal response. Therefore, it would appear that under the conditions of these cross-circulation experiments, the adrenals responded to the quantity of ACTH released rather than to the duration of their exposure to changes in the concentration of ACTH in the blood.

The specificity of the adrenal ascorbic acid depletion test for estimation of ACTH in blood by means of cross circulation was determined in another

series of experiments. Hypophysectomized Donors and Recipients were prepared in the usual manner and blood volumes ranging from 20 ml. to 40 ml. were transfused from one animal to another. At the appropriate time after termination of the cross-circulation, the second adrenal was removed from each and the ascorbic acid value compared with that of the respective control adrenal removed prior to cross-transfusion. The results indicated that no adrenal ascorbic acid depletion occurred in response to the cross-transfusion of blood from two hypophysectomized animals. Therefore, it is

TABLE 2. EFFECT OF DURATION OF CROSS-CIRCULATION ON ADRENAL ASCORBIC ACID RESPONSE IN HYPOX. RATS. (20 ML. BLOOD X-CIRC.)

No. of expts.	*Duration of X-circ. min.*	*% Change in adrenal ascorbic acid of hypox. ± S.E.*	*P*
11	10-20	— 17.2 ± 2.22	> 0.5
9	21-30	— 16.9 ± 3.63	> 0.5
4	31-above	— 19.8 ± 2.58	> 0.5

apparent that the cross-circulation procedure itself does not contribute to the adrenal ascorbic acid response. The presence of a pituitary factor, undoubtedly ACTH, is essential and specific for the adrenal ascorbic acid depletion under the conditions of these experiments.

DISCUSSION

Many attempts have been made to measure ACTH in blood. In general, two types of extraction procedures have been utilized: those employing the acid-acetone plasma precipitation and those utilizing oxycellulose column elution of ACTH from plasma. The levels of ACTH activity reported by those workers using the oxycellulose technique[5, 14] are generally lower than those claimed by other workers using the acid-acetone procedure.[1, 8, 9] Gray and Parrott[8] have investigated the recovery by acid-acetone of ACTH added to plasma; almost complete recovery was obtained. However, they were unable to prepare consistently active extracts from whole blood by this method, nor were they able to quantitatively recover ACTH that was added to whole blood. In addition, although Parrott,[10] using the acid-acetone method, was able to demonstrate quantitative recovery of added ACTH, she was unable to obtain positive regression between doses of extract.

In the investigation reported here, an excellent dose-response relationship has been demonstrated under standardized conditions by the cross-circulation of known amounts of blood between normal and hypophysec-

tomized rats, using the change in adrenal ascorbic acid of the latter as the measure of the response. In these experiments 5 ml. of blood exerted no effect on the adrenal of the Recipient hypophysectomized partner, while 10 ml. gave a significant response. This is in agreement with the results obtained by J. Richards (unpublished results, cited by Sydnor and Sayers[9]), who found that 4-6 ml. of blood obtained from the abdominal aorta of intact rats failed to deplete the adrenal ascorbic acid of hypophysectomized rats.

It should be pointed out that it is in no way assumed that the blood levels of ACTH estimated in normal animals under the conditions of the cross-circulation technique are the same as those present in normal unoperated animals. It is evident that the operative procedure of cannulation involves a "stress" which will increase the blood ACTH level. This level has been designated "basal" under the experimental conditions employed. However, this "basal" secretion is quite constant, and alterations in the rate of secretion of ACTH above and below this value may be demonstrated upon application of various experimental procedures. These will be reported in another publication.

SUMMARY

The blood content of ACTH has been estimated by means of a cross-circulation technique. If a normal rat is united with a hypophysectomized animal, the amount of ACTH in the blood of the intact rat can be estimated by the change produced in the adrenal ascorbic acid of the hypophysectomized partner. One adrenal is removed from the hypophysectomized Recipient before the cross-circulation and the second gland is removed after a known quantity of blood has been cross-circulated. By this method, a linear relationship has been found between the logarithm of the volume of blood transferred from the intact rat and the fall in adrenal ascorbic acid of the hypophysectomized animal. The ACTH present in 10 ml. of blood of a normal animal can be detected by this method.

ACKNOWLEDGMENT

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EVENTS

NU SIGMA NU LECTURE

March 7, 1950

MECHANISMS OF PULMONARY EDEMA. By Mark D. Altschule, Harvard Medical School, Boston, Massachusetts.

Many discrepancies between the left ventricular failure hypothesis of pulmonary edema and clinical observations suggest that this mechanism is not the entire explanation of this common clinical syndrome. For instance, pulmonary edema is less frequent in the aortic stenosis than in aortic insufficiency, yet the strain on the left ventricle is as great in the former. Again, the prolonged left ventricular strain of chronic hypertension rarely produces edema in contrast to its common occurrence in sudden hypertension. In the left-sided failure theory the rise in pulmonary capillary pressure determines edema formation. In mitral stenosis, however, pulmonary capillary pressures of 60 mm. Hg probably exist, yet in its uncomplicated form this clinical entity remains comparatively free from pulmonary edema.

An alternative explanation of the experimental and clinical observations takes into account the cumulative effects of many mechanisms. These may conveniently be divided into those which increase the amount of transudate, those which inhibit the reabsorption of the fluid, and those which tend to increase the total body water. Decreased plasma protein levels, increased pulmonary blood flow and raised capillary pressure certainly increase transudation. Epinephrine injection in animals can cause pulmonary edema; exponents of the left ventricular failure theory offer this as inferential evidence that hypertension in man may cause enough left-sided strain to produce edema. The isolation of abundant histamine from animal and human lung tissue provides a more logical relationship. Epinephrine is known to release histamine, and, experimentally, histamine can be shown to cause venous constriction and, perhaps, increased capillary permeability. Both of these latter may contribute to edema formation in man.

Pulmonary edema secondary to hypothalamic lesions in cats and following skull fractures in man may indicate central nervous and reflex mechanisms. The laboratory observation that sudden carotid sinus distension produces pulmonary venoconstriction provides a good explanation for the increased incidence of pulmonary edema in aortic insufficiency and acute hypertension. In chronic hypertension and aortic stenosis this distension is neither so sudden nor so great. Bronchospasm, in an unexplained manner, also contributes to transudate formation.

The experiments of Drinker indicate the highly important rôle of pulmonary lymphatics in reabsorption of pulmonary transudates. General cardiac decompensation manifest by increased systemic venous pressure may contribute to edema formation as much by decreasing lymphatic flow and thereby decreasing transudate reabsorption as by increasing pulmonary capillary pressure. The accumulation of excess body water, as in anemia, nephrosis, or beri-beri, also contributes to edema formation.

These and other mechanisms may be involved; no one factor is enough but the combined effects of many probably tip the balance in favor of pulmonary edema. Fortunately, treatment of this condition differs little no matter which explanation one adopts. Acceptance of this multi-factorial reasoning does, however, militate for the use of digitalis only in those cases of pulmonary edema in which heart failure plays a significant rôle.

R. I. B.

YALE MEDICAL SOCIETY

March 12, 1956

Studies in Extracorporeal Circulation with an Artificial Heart Lung Apparatus. By Bert K. O. Kusserow, Research Fellow in Pathology, Yale University School of Medicine.

The heart and lungs may be temporarily excluded from the circulation, either totally or partially, by the substitution of an apparatus which consists of a pumping component and an oxygenating component. Blood is withdrawn from the subject's venous system, delivered to the oxygenator, and returned to the arterial system by a balanced output double perfusion pump. The latter is a single diaphragm, double chambered pumping unit which functions simultaneously as withdrawal and return pumps whose outputs are identical. This characteristic prevents any imbalance in the subject's blood volume during perfusion. The pump valves operate on the principle of modified stopcocks.

Oxygenation and carbon dioxide removal are effected by the injection of tiny oxygen bubbles directly into the blood as it courses through the oxygenating component. The bubbles are produced by passing oxygen through a microporous porcelain filter. Excess oxygen and carbon dioxide are removed from the blood by the action of a constant delivery, slowly spinning centrifuge.

The apparatus has substituted for the respiratory, or cardiorespiratory, function of sixteen dogs for periods up to forty-three minutes. Nine of the last thirteen dogs have been permanent survivers.

B. K. O. KUSSEROW

Chromosomal Sex Differentiation by Cytological Techniques. By Walter Herrmann, Department of Obstetrics and Gynecology, Yale University School of Medicine.

Chromosomal sex may be determined from cheek scrapings. A discoid intranuclear chromatin mass adjacent to the nuclear membrane appears in 0-4 per cent of the cells of men and 10-32 per cent of the cells of women. In one patient with adrenal hyperplasia and two with gonadal dysgenesis, anatomical sex was shown to differ from chromosomal sex.

J. P. G.

PHYSIOLOGY SEMINAR

March 12, 1956

PHYSIOLOGICAL CHANGES IN THE CIRCULATION AT BIRTH. By Geoffrey S. Dawes, Director of the Nuffield Institute for Medical Research, Oxford, England.

The fetal circulation of sheep, obtained by cesarean section, has been studied for the purposes of elucidating the factors regulating blood flow through the placenta, the changes in blood flow through the lungs at birth, the closure of the ductus arteriosus, and the relationship of oxygen consumption to the partial pressure of oxygen in the blood.

The autonomic nervous system rather than the placenta appears to be responsible for controlling the placental blood flow. Full-term pregnancy in sheep lasts 147 days. Between the 90th and 100th day, the autonomic nervous system begins to function. At this time, but not before, the fetus responds to anoxia by an increase in the blood pressure; this, in turn, increases the blood flow through the umbilical vessels. The response to anoxia increases progressively as the fetus approaches birth. These relationships are species specific and are not applicable to the rabbit and certain other species in which the autonomic nervous system is poorly developed before birth.

During the fetal period, only ten per cent of the ventricular output is pumped through the lungs. At birth the pulmonary blood flow increases ten- to twenty-fold. The mechanism responsible for this change is primarily mechanical, rather than nervous or humoral. Perfusion experiments on isolated fetal lungs indicate that distention of the lungs with a gas is of "paramount importance." While the density of the gas may produce variations in the pulmonary resistance and blood flow, this appears to be a minor factor.

Two mechanisms have been elucidated for the constriction of the ductus arteriosus. The criteria employed for ductus closure in the experiments to be discussed include the disappearance of the characteristic presystolic murmur, electrocardiographic reversion to normal pattern, and actual reduction in the external diameter of the ductus arteriosus. In one group of experiments, closure of the ductus is obtained by raising the oxygen saturation in the carotid arteries from that normally present (72%-80%) to about 95%. In another series of experiments, closure is obtained by subjecting the fetus to a short period of asphyxia. This procedure is attained by an increase in blood pressure which is mediated by the autonomic system (as discussed above). The blood pressure effect is apparently of significance inasmuch as the sympathetic amines also produce constriction of the ductus arteriosus. It is of interest that while the rôle of an increased blood oxygen saturation may be difficult to explain in ductus closure, the rôle of asphyxia may be related to the period during which the umbilical cord is separated from the placenta at birth.

In the fetal sheep, a reduction in the oxygen saturation of blood is attended by a corresponding reduction in oxygen consumption. While

studies in human babies and in puppies show a similar relationship, it has been known in the past that the adults of these species do not demonstrate any relationship between oxygen consumption and blood oxygen saturation level. The significance of this phenomenon is, as yet, a matter of conjecture; it is of particular interest in relation to the very low values of oxygen saturation normally present in the human fetus.

STANLEY HARRIS

ALPHA OMEGA ALPHA ANNUAL LECTURE

March 13, 1956

AN EXPERIMENTAL ANALYSIS OF THE DEFENSE OF THE HOST IN ACUTE BACTERIAL PNEUMONIA. By Barry Wood, Johns Hopkins University, Baltimore, Maryland.

Aspects of the problem of host defense in pneumococcus infection include: histopathology of lesion, inflammatory response, operation of cellular defense, lines of cellular defense, and relationship of cellular defense to curative action of penicillin.

The lesion can be divided into several zones. The outermost is the edema zone in which bacteria multiply rapidly. Central to this are the zones of early and late consolidation in which the alveoli are filled with leukocytes, and the zone of resolution with macrophages predominating and cocci in resting metabolic stage.

Using transparent chambers inserted into the ears of rabbits, the development of the inflammatory response can be observed. The phagocytes adhere to the capillary walls, first to the side nearer the inflammation, later to the entire wall of the vessel. The leukocytes then leave the vessel by diapedesis and accumulate at the site of infection.

Phagocytosis is lethal to pneumococci, the bacteria surviving only fifteen to thirty minutes within the leukocyte. At one time it was thought that phagocytosis occurred only in the presence of host antibodies when the capsule of the pneumococcus became coated with gamma globulin. It has now been shown, however, both *in vitro* and *in vivo* that some phagocytosis occurs in the absence of antibodies. In such cases, the leukocyte traps the bacterium between itself and another surface. Surfaces which may be used in this way include the wall of the alveolus, neighboring phagocytes, and fibrin networks found in the exudate.

In pneumococcus infections, bacteria may invade the lymph nodes and blood stream. Phagocytosis in the absence of antibodies occurs in these systems as well as in the lung.

Penicillin is bactericidal only when the cocci are in an active metabolic phase. Therefore, penicillin is effective in the edema zone, but ineffective in the center of the lesion.

Problems for future research involve the mechanism of phagocytic adherence to capillary walls, believed to be related to fibrinogen accumula-

tion, and the nature of the anti-phagocytic action of the pneumococcus capsule.

LISA STEINER

GASTROINTESTINAL STUDY UNIT

March 15, 1956

MODERN CONCEPTS OF SPRUE. By Chester M. Jones, Clinical Professor of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston.

Tropical and non-tropical sprue present similar clinical pictures and are very probably the same disease. The frequent occurrence of a folic acid or vitamin B_{12} correctable macrocytic, hypochromic anemia in the former, as opposed to its less frequent, more refractory counterpart in the latter does not constitute sufficient evidence for considering these as separate entities.

Although no histologic pathology is demonstrable, the basic lesion in this disease is thought to be at the cellular level and causes a decreased absorption of fat, carbohydrate, protein and vitamins by the small bowel. The resulting sprue syndrome consists clinically of fatigue, frequent large bowel movements, weight loss, distension, stomatitis and cutaneous pigmentation. Laboratory evaluation frequently reveals fatty stools, decreased serum calcium and prothrombin time, and a flat oral glucose tolerance curve. Barium swallow studies characteristically show puddling and dilated loops in the small bowel.

Cortisone markedly improves absorption from the small bowel in the sprue syndrome. A large number of patients treated with steroids have shown complete reversal of the disease process, though long term results are not as yet available. Some patients have shown improvement on a gluten free diet.

J. C. C.

ANESTHESIOLOGY SEMINAR

March 26, 1956

ANESTHETICS AND ADRENAL FUNCTION. By Leroy D. Vandam, Department of Anesthesiology, Harvard Medical School, Boston, Massachusetts.

During diethyl ether anesthesia in both man and dog, serum levels of 17-hydroxycorticoids increase and continue to do so for several days after operation. The initial increase is due to the anesthesia, the continuing rise to surgical trauma. The serum levels reflect not only enhanced secretion but decreased destruction and/or excretion of the steroids; this was shown by observing increased levels of serum 17-hydroxycorticoids during anesthesia and surgery on adrenalectomized dogs maintained on adrenal cortical extract. During anesthesia and surgery under hypothermic conditions, the increase in serum 17-hydroxycorticoids is minimal, but the levels increase markedly as normal body temperature is reached and continue high for several days.

In the intact dog ether anesthesia increases cardiac output, but after sympathectomy and adrenalectomy, cardiac output and arterial pressure fall while right auricular pressure rises. Thus, ether anesthesia appears to stimulate the secretion of epinephrine and norepinephrine.

The fear and starvation that precede surgery also stimulate the secretion of epinephrine and norepinephrine. But these emotions do not appear to stimulate the adrenal cortex, for patients who are prepared for surgery but not operated upon show no increase in 17-hydroxycorticoids.

J. P. G.

YALE MEDICAL SOCIETY

April 9, 1956

ATYPICAL HYPOPITUITARISM. By Harold Sheehan, Professor of Pathology, Liverpool University, Liverpool, England.

A carefully selected series of 100 cases of postpartum pituitary necrosis, studied clinically and pathologically, was the basis of a study comparing the extent of the hypophyseal lesion with the clinical course. Patients were grouped on the basis of the percentage of the pituitary tissue lost. It was ascertained that adjacent hypothalmic and neurohypophyseal regions were intact and no aberrant pituitary tissue was present. The clinical classification was based on three salient features. The first related to the persistence of function of a particular target organ. The second group showed unusual deficiency of a target organ. In the third group there was a complex picture of hormonal interdependence which produced symptomatology related to marked atrophy of one target organ, but which responded to replacement therapy with hormone of another target organ. Where possible, studies of target organ function and histology were carried out.

Certain women after a typical episode of postpartum pituitary necrosis maintained ovarian function as evidenced by occasional uterine bleeding, typical menstruation, or subsequent pregnancy. The ovaries lacked Graafian follicles but the primordial ova remained. No correlation was found between the extent of pituitary damage and continued ovarian function. The persistence of target organ function is perhaps produced by a minimal production of tropic hormone which is stored and periodically released. Alternatively, it has been postulated that the persistent functioning of a target organ may be related to a preponderance of cells producing its tropic hormone in the remaining pituitary tissue. This is unsupported by histological evidence.

Extreme target organ atrophy was studied in the thyroid and adrenals. Severe thyroprivic and adrenal insufficiency states were noted in these cases. Again there was no correlation between the extent of damage to the pituitary and target organ function. These instances may represent people who normally had little reserve in a particular target organ so that removal of the tropic hormone led to severe failure of function with concommitant atrophy. The picture is complicated by cases which showed no functioning target organ tissue on clinical testing but failed to develop severe symptoms referrable to that organ.

Most confusing were those cases where symptomatology was primarily related to one target organ but responded to therapy with the hormone of another, as in secondary myxedema controlled by cortisone. These cases sharply illustrate the lack of information concerning the intricacies of the hormonal interrelationships of the pituitary target organs.

ROBERT S. NEUWIRTH

ANATOMY SEMINAR

April 17, 1956

THE FINE STRUCTURE OF THE CORNEA. By Marie A. Jakus, The Retina Foundation, Boston, Massachusetts.

An electron microscopic study of the cornea has disclosed a complex fine structure.

The most superficial layer of the corneal stratified epithelium consists of a pavement of squamous cells which interdigitate with one another by means of stubby cytoplasmic processes, like fasteners in a zipper. The comparative looseness of the "zippering" in this layer suggests that the cells are rather readily desquamated. Except for fine filaments, the cytoplasm of the squamous cells is not remarkable. In the embryonic chick cornea, however, the undifferentiated cytoplasm of these cells is filled with granular endoplasmic reticulum, now identified with the ergastoplasm of light microscopy. The nucleus of the adult squamous cell appears as a lobulated, ovoid aggregation of electron opaque granules, bounded by a double membrane. The long axis of the nuclear profile is roughly parallel to the plane of the squamous cell. Deep to the squamous pavement is the intermediate layer of cells. The larger cytoplasmic processes of these cells probably effect a tighter "zippering" than occurs in the squamous layer. The nucleus of the intermediate cell resembles that of the squame. The stratified epithelium is completed by a basal layer of tightly "zippered" round cells containing lobulated, spheroid nuclei.

A thin band of moderate electron opacity, seen beneath the basal cells, represents the basement membrane of the epithelium. Occasionally this membrane displays a finely fibrillar or lamellar architecture. On the surface of the membrane subjacent to the basal epithelial layer, a regular granulation or pseudo-striation is visible.

Bowman's membrane, an essentially homogeneous band in the light microscope, is revealed in the electron microscope as a feltwork of randomly oriented filaments. In favorable preparations the filaments show a periodicity approximating that of the collagen fibers in the corneal stroma. The appearance of periodicity in these filaments is frequently obscured, perhaps by absorbed substances of appropriate electron opacity, presumably containing mucopolysaccharide. The filaments measure about 180-190 Å in diameter in the electron micrographs, some 40-50 Å narrower than the stromal collagen fibers.

Beneath the membrane of Bowman lies the corneal stroma, composed of lamellae of collagen fibers. The fibers within any lamella are parallel to the

plane of the corneal surface and to each other; thus, each fibrous lamella may be said to have one axis of orientation to which its constituent fibers are essentially parallel. Since in alternate lamellae these axes of orientation are perpendicular to each other, a criss-cross pattern occurs among the lamella. The diameters of the intralamellar collagen fibers are characteristic for species (man 230 A; rat 270-275 A; mouse 370-375 A). Scattered among the fibers, and occasionally adherent to them, is a fine, filamentous component, thought to contain elastin or mucopolysaccharide.

Descemet's membrane comprises an unusual arrangement of collagen. Indeed, before the preparation of tangential sections of the membrane a satisfactory interpretation of its fine structure from transverse sections was wanting. Descemet's membrane consists of a pile of bidimensional grids of collagen laid parallel to the plane of the corneal surface. Each bidimensional grid is a mosaic of repeating hexagonal units. The corners of each hexagonal unit are marked by dense nodes connected by internodal fibers which, therefore, describe the perimeter of the hexagon. In the center of each hexagon is a single node, connected by radial fibers to the six peripheral nodes at the corners of the hexagon. The grids formed by the repetition of the hexagonal unit are piled so that the corresponding nodes and internodal fibers of all grid layers are in register. (From this description one may envision the original problem in interpretation from transverse sections and its clarification by the examination of tangential sections.)

The deepest stratum of the cornea, a single layer of "zippered" endothelial cells, provides a comparatively plain limiting wall for a tissue of intricate internal architecture.

ALLAN H. MILOFSKY

YALE MEDICAL SOCIETY

May 14, 1956

ACUTE HYPERTENSION IN THE PULMONARY VASCULAR BED OF THE DOG: A PHYSIOLOGICAL STUDY. By S. E. Downing, Fourth year Student, Yale University School of Medicine.

Although a rather extensive literature on the subject of pressoreceptors on the pulmonary vascular bed has evolved, there is a rather wide divergence of opinion regarding the nature of the reflex response resulting from induced pulmonary hypertension.

In general, the present approach to the problem is to perfuse the left lung of the intact dog with normal saline at body temperature under variable pressures and rates of flow and to measure simultaneously the perfusion pressure and rate of flow, the systemic pressure, the pulse rate, the main pulmonary arterial pressure, and the respiratory rate and depth.

An apparatus was constructed by means of which the perfusion pressure and rate of flow could be regulated and recorded.

In order that the experiments might be performed on the intact animal, it was necessary to construct various cannulae so that the manipulations could be performed on animals with intact chests. Consequently, into each

animal an infusion cannula was placed in the left pulmonary artery, a guide cannula sutured to the main pulmonary artery through which an L.P. needle could be passed for the purpose of measuring the main pulmonary arterial pressure, and two specially designed snares for the pulmonary veins, one for the upper and middle lobe veins and one for the lower lobe veins, by means of which the venous return of the left lung could be occluded or opened at will from the exterior of the animal.

Two groups of animals were prepared surgically, acute and chronic. Veratridine was used to demonstrate the presence of intact nerve pathways from the pulmonary vascular bed which might have been damaged at the time of surgical preparation.

The results of this study demonstrated that the response of the intact dog to acute hypertension in the peripheral pulmonary vascular bed consists of three components: (i) a drop in systemic pressure, (ii) bradycardia, and (iii) a change in the respiratory pattern. All of the components had their onset simultaneously about six or seven seconds after beginning the perfusion. None of the components were observed to occur following ipsilateral vagotomy. The fact that the response does not occur when the pulmonary vein snares are open, but does readily with the snares closed would suggest that the majority of the receptors are located on the venous side of the pulmonary circuit.

S. E. DOWNING

A Study of Some Aspects of Fatty Acid Metabolism in Man. By Robert McDivitt, Fourth year student, Yale University School of Medicine.

Five normal humans without evidence of metabolic disease were given 200 micro curies of acetate $1\text{-}C^{14}$ each, and blood samples were drawn at intervals up to 96 hours. The plasma lipids were separated into triglycerides, cholesterol esters, and phospholipids with a modified form of Fillerup and Mead's method of silicic acid chromatography. Following this, an aliquot from each fraction was saponified and the amount of radioactivity in each fatty acid sample determined, using a liquid scintillation counter. Further aliquots of fatty acids from each triglyceride, phospholipid, and cholesterol ester fraction were chromatographed on hyflow-supercel reverse phase columns to effect separation of individual fatty acids. It was found that incorporation of radioactivity was much greater and more rapid in the triglyceride fatty acid fraction than in either the phospholipid or the cholesterol ester fatty acid fractions, peak specific activity of this fraction occurring within two hours after administration of the acetate. The maximum specific activity of the phospholipid fatty acid fraction occurred at 12 hours, while that of the cholesterol ester fatty acid fraction occurred between 24-48 hours. It was further observed that the greatest concentration of stearic and other saturated fatty acids occurred in the triglyceride fraction, while the unsaturated fatty acids were found in greatest concentration in the cholesterol ester fraction. Possible correlation between fatty acid composition of these compounds and difference in rates of incorporation of radioactivity from acetate $1\text{-}C^{14}$ was discussed.

ROBERT MCDIVITT

BOOK REVIEWS

INTEGRATED GYNECOLOGY. PRINCIPLES AND PRACTICE. By I. C. Rubin and Josef Novak. New York, McGraw-Hill Book Company, Inc., 1956. Vol. I, 589 pp., Vol. II, 496 pp., Vol. III, 682 pp. $60.00.

These three handsome volumes, written in the European tradition, represent a commendable and ambitious undertaking, original in that considerable effort is made to integrate gynecology with the broader aspects of medicine.

Volume I commences with chapters on the anatomy and evolution of the female pelvic organs with considerably more extensive accounts than are found in most texts. The chapters on physiology, including endocrinology, and on gynecological diagnostic procedures are also very comprehensive. The section on tubal insufflation and hysterosalpingography is, needless to say, authoritative. It seems a little surprising that a book published in 1956 should still see merit in the Richardson pregnancy or sex differentiation tests. There follow sections on congenital anomalies and a listing of mechanical and chemical injuries (some of which are only distantly related to the female reproductive system). Lengthy descriptions of various displacements of the pelvic viscera precede a chapter on infections.

Volume II classifies the various tumors of the female sex organs with very complete pathological descriptions and illustrations. The microphotographs are of variable quality, but on the whole demonstrate the pathological processes satisfactorily. The volume also covers constitutional disorders and problems of reproduction, including a competent section on sterility.

Volume III commences with a chapter on the relationship between reproductive and other body systems. Unfortunately, the effort is made to list, rather than sort, the important from the unimportant. For example, a full page is devoted to the rôle of the nose in dysmenorrhea and labor pains, but one sentence covers German measles in relation to pregnancy and two cover the rôle of oxygen in retrolental fibroplasia. There is no discussion of the pre- and post-operative care of the diabetic, or mention of the relationship of diabetes to endometrial carcinoma. The breast and urinary tract are regarded as an intimate part of gynecology and are covered rather extensively. Pelvic bowel lesions are, in comparison, slighted. The chapter on gynecological symptomatology is a little redundant with terms such as "proiopolymenorrhea," "algomenorrhea," "oligo-opsomenorrhea," "fluor," and others. There is a good collection of operative illustrations in the chapter on operative gynecology. However, there is unwarranted support given to subtotal hysterectomy; the Witzel enterostomy is recommended for intestinal distention or for peritonitis; and the implication that lymph node dissection is not always necessary in the Wertheim operation is supported by the surprising statement that "it became clear that lymph nodes can destroy cancer cells." The chapter on physiotherapy is adequate in its coverage of Dominici tubes, Grenz rays, pressure and suction therapy, and spas, but there is no real discussion of modern radium and x-ray dosimetry, supravoltage therapy, radioactive gold, cobalt, or other isotopes.

The three-volume set is designed for students and general practitioners. To be a suitable textbook, it might have to undergo considerable editing. Its cost and length, (1,767 pages, including some 231 pages of bibliography) seem in part to be the result of a literary verbosity. It seems unnecessary to point out, even to the medical student, that women are less prone to baldness than men and do not ordinarily grow beards (Vol. I, p. 163; Vol. II, p. 350; Vol. III, p. 37), or to illustrate the fat and the thin woman. Although there is much to be said for the historical approach, too much space is taken up with accounts of methods and ideas which are now abandoned. There are also quite a number of statements of fact, such as that Stein-Leventhal ovaries are congenital or that radiation of the spleen is useful in uterine hemorrhage, which are, at best, open to question.

The organizational plan of the three volumes is good, in spite of some repetition in the text. The references, especially to the German literature during the earlier part of this century, are very extensive. Rather than a text, it is a useful reference work and it represents a significant addition to the gynecological literature.

JOHN MCLEAN MORRIS

TOPLEY AND WILSON'S PRINCIPLES OF BACTERIOLOGY AND IMMUNITY. 4th ed., revised by G. S. Wilson and A. A. Miles. Baltimore, The Williams and Wilkins Co., 1955. viii, 2,332, xlviii pp. $24.50—the set of 2 volumes.

Since it first appeared in 1929, "Topley and Wilson" has been constantly at the side of very professor of medical bacteriology who was preparing a lecture and of many a bacteriologist doing actual work. Its authorative and comprehensive coverage and readable style have long set it apart from even the best of other texts in its fields. At intervals, revisions have been made to keep pace with the active progress of medical research.

In this, the first revision since 1946, the surviving author has again had the collaboration of Dr. A. A. Miles, Director of the Lister Institute, London. Their philosophy may be expressed by quoting from the preface to this edition:

". . . although the book was originally intended for the general student of medicine and biology, its character has gradually changed, till now this claim can no longer be justified. The great expansion of knowledge during the last twenty-five years has forced us to deal more and more with the bacteriology of infection in man and animals, and to treat this at increasingly greater length . . . much of interest to the non-medical bacteriologist has had to be omitted and a mass of detail has had to be included so as to render parts of the text frankly unsuited for the undergraduate medical student. We hope it will prove of correspondingly greater value to the postgraduate student of human and veterinary medicine, to the teacher of bacteriology and immunity and to the research worker."

Although the text is some 277 pages longer than that of the third edition, the new matter added is even more extensive. This appears to have been achieved, in part, by summarizing less important material formerly treated

at length and, in part, by judicious pruning of the fine leisurely sentences composed at a time when type costs were not so high. A detailed comparison of several representative chapters with those of the third edition arouses admiration for the skill—and even devotion—with which this revision has been made. There is not a sentence of added material which is not in good context, or which has the aroma of the scissors and paste-pot school of revision. Many of the sections, especially those dealing with metabolism, phage, and chemotherapy have been completely rewritten, as the newer research provided a fresh approach to the presentation. As might be expected, the more settled areas, such as descriptions of organisms, have merely been brought up to date.

Inevitably in a work of this magnitude minor faults can be found. The space devoted to the general properties of viruses still seems inadequate, in comparison with that devoted to some less important topics. The discussion on photometric assay methodology has been shortened (to the point of omitting the senior author's own work!) although laboratory applications are ever increasing. The discussion on probit assay (p. 1133) would deserve more extended treatment—or at least a modern reference, such as to Finney's book—and the graph (Fig. 242) illustrating it is inaccurate if employing a linear ordinate. The references—there are often several hundred per chapter—are a most valuable feature of the book. They stress, rightly, early contributions and original sources. Nevertheless, the reader might well appreciate knowing of certain recent monographs, symposia, and review articles which provide useful second-level introductions to special topics. Examples of this are Waksman's monograph on actinomycetes, van Heyningen's on toxins, and the recent New York Academy of Sciences Symposium on hyaluronic acid systems.

Nevertheless, we are deeply grateful to the authors for this fine revision of a classic. It represents a substantial addition to what we have had available.

HENRY P. TREFFERS

ANDROGENS: BIOCHEMISTRY, PHYSIOLOGY AND CLINICAL SIGNIFICANCE. By Ralph I. Dorfman and Reginald A. Shipley. New York, John Wiley and Sons, 1956. xvii, 590 pp. $13.50.

This exhaustive discussion of androgenic steroids and their metabolic relatives is divided into the biochemistry (with considerable discussion of intermediate steroid metabolism), the physiology, and the clinical applications of these basic disciplines. The discussion of biochemistry, which occupies 140 pages, presents a very complete discussion of the steroid chemistry, intermediary metabolism, and present knowledge of the sources and pathways by which the various steroids ultimately arrive in the urine. This discussion assumes no initial knowledge on the part of the reader and takes him stepwise from the most elementary to the most advanced concepts. A similar exhaustive attack is made on the physiological and clinical departments of the book. Although some small quibbles might be raised with regard to the particular assay methods discussed in the methodological

appendix, over-all this volume deserves nothing but praise. It is an absolute "must" for anyone who enters this complicated field. Moreover, the organization of the book is such that the casual reader seeking an isolated bit of information will be as well served as he who sits and studies it intensively. The book is highly recommended to anyone whose interests include an understanding of the biochemistry, physiology, or clinical aspects of the androgenic steroids.

PHILIP K. BONDY

BONE: AN INTRODUCTION TO THE PHYSIOLOGY OF SKELETAL TISSUE. By Franklin C. McLean and Marshall R. Urist. Chicago, University of Chicago Press, 1955. xii, 182 pp. $6.00.

The authors have produced a well-organized review of skeletal tissue. It is taken up in a logical sequence beginning with bone as a tissue going through the histogenesis, chemical composition, process of calcification and absorption, and the effects of enzymes and hormones on bone. Following this there is a chapter on mineral metabolism, a chapter on radio isotopes, and a discussion of post-fetal osteogenesis. Finally, in the light of the basic discussions listed previously, the problems related to the healing of fractures and the pathological physiology of bone are discussed. This book can be recommended as fulfilling its title, "Bone—An Introduction to the Physiology of Skeletal Tissue" very adequately. It is recommended to anyone interested in the subject.

CHARLES O. RECHTOL

ALCOHOLISM—ITS PSYCHOLOGY AND CURE. By Frederick B. Rea. New York, The Philosophical Library, Inc., 1956. 143 pp. $3.50.

This book may enlighten those readers who have been unaware of the seriousness of alcoholism as a public health problem, or who have censured alcoholism as willful and self-induced immorality, or who have regarded alcoholism solely as a physiological disease, and those who have been unaware of the successes of the Alcoholics Anonymous movement.

After rejecting the concept of alcoholism as a physiological illness, Mr. Rea presents evidence that alcohol addiction also cannot be understood solely as a psychological illness. He proposes that alcoholics really are sinners who cannot resist their sinning unless they experience a spiritual and moral conversion to sobriety such as is possible through affiliation with Alcoholics Anonymous. His main argument seems to be that psychotherapists are less successful in treating alcoholics than are members of Alcoholics Anonymous.

Mr. Rea errs in excluding disorders of personality and character from the category of psychological illness. He also errs in excluding the recovery of alcoholics via Alcoholics Anonymous from the category of successful psychotherapeutic experience. Psychotherapy occurs whenever a person or group tries to treat sick people through the influence of their relationship. The psychotherapy may not be explicit or formalized, and the therapists

may not conceptualize themselves as such, nor be qualified by training, experience, or accreditation. Nonetheless, if the relationship is therapy-oriented, involves sustained personal interaction and permits corrective emotional experiences, the situation is a psychotherapeutic one. This would apply to the alcoholic interacting with Alcoholics Anonymous members as well as it would to the analysand interacting with his psychoanalyst.

Alcoholics Anonymous has not cured alcoholics primarily because of spiritual or moral conversion, or because "only an alcoholic can help an alcoholic." Rather, it is that its members are willing and able to provide the intimate individual and group psychotherapeutic relationships needed to effect recovery in many of their fellow sufferers. This activity in itself sustains and facilitates their own recovery. Despite its lack of a rational frame of reference, Alcoholics Anonymous has contributed successfully to the pressing social need for psychotherapists for alcohol addicts.

LOUIS B. FIERMAN

ESSAYS IN BIOCHEMISTRY. Samuel Graff, Ed. New York, John Wiley and Sons, 1956. x, 345 pp. $6.50.

This is a Festschrift honoring Hans Thacher Clarke on his retirement from Columbia University. Each of the 25 essays, written by different authors (or pairs of authors), differs as much in style and manner of presentation as it does in subject matter. Some of the articles are informal, almost conversational, more nearly like nonexhaustive reviews; others are like the conventional experimental reports. The high level of scholarship maintained throughout more than justifies the unusual amount of freedom given the authors. All essays are authoritative, lucid, and highly provocative, often sparkling with fresh speculations. Work published only last year and some yet unpublished is included in many of the discussions and gives the book a quality of immediacy.

The essays average 14 pages in length, varying from 15 to 21 pages, and appear alphabetically in order of the authors' names. All but one contains references to the literature, and the volume has an index. There is something to interest almost everyone. The contents, with the authors' names in parentheses, follow: metabolic products of basidiomycetes (Anchel); heterogeneity of DNA (Bendich); biosynthesis of branched-chain compounds (Block), of peptide bonds (Fruton), of porphyrins (Shemin), of aromatic compounds (Sprinson); lysogeny (Borek); development of a plasma volume expander (the Bovarnicks); conjugated proteins (Chargaff); thymine (Cohen), lipide (Gurin), and inositol (Magasanik) metabolism; steroid hormones (Engel); bacterial viruses (Evans); cancer (Graff); tetrazoles (Herbst); asymmetric reactions (Hirschmann); nitrogen (Hoberman) and glycogen (the Stettens) turnover; ferritin (Mazur); nitrogen transfer (Ratner); mechanism of enzyme action (Rittenberg); protein structure (Stein); veratrum alkamines (Wintersteiner); transforming phenomenon (Zamenhof).

J. P. G.

POLIOMYELITIS. Papers and Discussions Presented at the Third International Poliomyelitis Conference. Philadelphia, J. B. Lippincott Co., 1955. xxiv, 567 pp.

This volume contains the twenty-six papers and the discussions which followed them as well as the twenty exhibits presented at the Third International Poliomyelitis Conference in Rome in 1954. The field was covered under the headings: Social Aspects, Acute Medical Care, Infection and Immunity, Developments in Tissue Culture, Orthopedics, Physical Medicine and Rehabilitation, and, finally, Trends in Poliomyelitis. Current concepts of these subjects were presented by eminent authorities from various parts of the world; the result is a timely and useful book for all interested in poliomyelitis, from whatever point of view.

The sessions on clinical problems contain excellent papers on the diagnosis and treatment of the acute disease. Outstanding is the paper by Hodes, "Treatment of Respiratory Difficulty in Poliomyelitis," a clear and concise analysis of this most complicated subject in terms of newer concepts of respiratory physiology. Excellent discussions on orthopedic problems and rehabilitation form another valuable section, exceptionally well illustrated.

Advances in the virology and immunology of poliomyelitis have occurred with extraordinary rapidity since the introduction of tissue culture techniques in 1949. Dr. John Enders reviewed the recent developments in tissue culture and their application in relation to studies on the epidemiology and pathogenesis of poliomyelitis were discussed in a number of papers. Papers by Salk and Sabin presented different approaches to the problem of active immunization. Dr. Salk reviewed his experience with formalinized vaccine, and Dr. Sabin presented the case for attenuated live virus vaccines. The theoretical considerations behind both approaches received thorough and interesting exposition.

Much of the material presented in this book has appeared elsewhere, but it is valuable to have it brought together as a summary of poliomyelitis as a world problem in what will probably soon be classified as the pre vaccination era in the history of the disease.

DOROTHY M. HORSTMANN

RHEUMATOID ARTHRITIS AND PSORIASIS VULGARIS. By Tibor Benedek. Ann Arbor, Edward Brothers, Inc., 1955. 308 pp.

More than moderate attention has been given to the reading of this book and to the wording of its review. Dr. Benedek supplies the etiology (*B. endoparasiticus* Benedek) and treatment (specific vaccine) of two diseases which have been and still are under active study. The author regards psoriasis vulgaris as one of the external or cutaneous manifestations while rheumatoid arthritis is the internal allergic response to this organism. Investigators in these fields are yet trying to gain a foothold. It is probably quite accurate to say that most believe that sensitization of tissues is involved in both illnesses. The combination of psoriasis and arthritis is well recognized but all rheumatologists are not prepared to call this inflammation of joints, *rheumatoid arthritis*.

The situation thus makes an adequate appraisal of the book difficult since much depends on a general confirmation of the author's results. I am in no position now to support or refute his findings and I know of no one who is.

In an effort to get on more firm ground, consideration was given to the other aspects of the book while the matter of etiology was kept open. It was felt that Dr. Benedek might have an advantage in being able to sort much of the accumulated data on psoriasis and arthritis in a manner that has not been available under existing circumstances. We, like most others, must use these studies for selection and revaluation of approach and can disregard little with any finality. The assumption of etiology is not helpful in this respect, probably because the prevailing view of etiology though hazy is not unlike that of the author. In other words, much remains unclear, and apparent inconsistencies must still be discarded as extraneous rather than explained.

The book by its nature is not detailed enough for the clinician, and the list of references has not the broadness of scope which a student with an investigative interest will need. The material is presented clearly and with emphasis by repetition. There are good illustrations. This monograph may be of interest to those who are more actively concerned with etiology and therapy. Familiarity with both the clinical aspects of these illnesses and the background literature places them in a position to decide whether the work of Dr. Benedek is convincing enough for confirmation or whether it serves to suggest some change in their own approach.

This Clinic will include Dr. Benedek's studies on the grounds that it is a mistake to disregard what may be the obvious. There are only two ways of establishing the merit of his work, and of these the simpler and more direct is repetition. Time is needed to place a value on this book.

PAUL L. BOISVERT

EXPERIMENTAL TUBERCULOSIS. Bacillus and Host (Ciba Foundation Symposium). G. E. W. Wolfstenholme, M. P. Cameron, and C. M. O'Connor, Eds. London, J. and A. Churchill; Boston, Little, Brown and Co., 1955. xii, 396 pp. $9.00.

This is a critical period for the research worker in tuberculosis. Great improvements in chemotherapy, surgery, and public health measures have resulted in a rapid decline in the death rate and the closing of some sanatorium facilities. What, then, could be more natural than for the uninformed to assume that most of the problems in this field have been solved, and that investigators should turn their attention to other more pressing medical questions. The fallacy of such reasoning is illustrated beautifully by this compact volume in which the reader learns on the one hand of the incompleteness of our knowledge and the complexities of the fundamental mechanisms involved in tuberculosis and, on the other hand, of the inroads that are currently being made in the understanding of many of these mechanisms. It becomes obvious that the recent successes of chemotherapy have not only facilitated clinical management of the patient, but have also

given a renewed impetus to research into the basic microbiological and pathogenetic processes.

The book presents the proceedings of a symposium on "The tubercle bacillus and the reactions of the host tissues" held in London in October 1954, and sponsored by the Ciba Foundation for the Promotion of International Cooperation in Medical and Chemical Research. Here were gathered a good many of the import research workers in tuberculosis and leprosy "quietly occupied with apparently recondite problems relating to the fundamental nature of the bacillus, and of the host reactions to it," to use the words of the chairman, Dr. Arnold Rich. Included in the topics discussed were chemistry of the tubercle bacillus, tissue reactions to the bacillus and to its components, problems related to hypersensitivity, immunity, and serology, the application of tissue culture methods, factors involved in the pathogenicity of the tubercle bacillus, the influence of steroid hormones, and others. In addition, there is an addendum on experimental leprosy in which many of the common problems in these two diseases are presented.

Each of the papers, of which there are 20, presents a wealth of material summarizing the author's own work in the field and usually discussing the results of others as well. Even more valuable, however, is the lengthy, uninhibited discussion following each paper which serves to amplify and clarify the experimental details as well as to bring to the fore many conflicting opinions among the experts.

The entire volume is well edited and should make interesting and important reading for research workers in tuberculosis and leprosy. For medical people concerned with the two diseases and for experimentalists in related fields, it will certainly provide insight into a good deal of the basic research, aside from chemotherapy, under active investigation today in this field.

EMANUEL WOLINSKY

PREVENTIVE MEDICINE IN WORLD WAR II. VOL. III. PERSONAL HEALTH MEASURES AND IMMUNIZATION. Washington, D. C., Office of the Surgeon General, 1955. xiv, 394 pp.

In World War II, the Medical Department of the United States Army developed and managed personal hygiene, sanitation, mental hygiene, nutrition, disease and injury prevention on a scale never previously necessary in United States military efforts. Consequently, the problems inherent in such an undertaking were numerous, and this book, which focuses on personal health measures, presents an objective analysis of these developments. This is the third volume of the trilogy of preventive medicine experiences in World War II; the two previous reports were concerned with epidemiology and environmental sanitation.

The chapters, presented by highly qualified analysts, are concerned with the military aspects of manpower selection, personal hygiene, clothing, nutrition, malnutrition and deficiency diseases, preventive psychiatry, accidental trauma, and the Army immunization program. Re-appraisal of policy

concerning each of these aspects was imperative throughout the progress of World War II and the mechanisms of dispersing current information regarding personal health measures were effective only insofar as they became highly individualized.

The military experiences in provision of adequate clothing and in maintaining optimum nutritional status, based on careful physiological observation, are especially well discussed. Preventive psychiatry based on statistical epidemiology appears to be feasible in view of the military psychiatric experience. Accidental trauma was conclusively proved to be one of the major mass health problems and this recognition is of fundamental importance as military units become increasingly mechanized. The Army immunization program is thoroughly discussed here, and its success is a tribute to the many professional, technical, and administrative workers without whose co-operation such a selective program would not have been accomplished.

This book will be an extremely useful reference text for any future consideration of fundamental problems related to mass population groups.

JAMES C. NIEDERMAN

Urology. By B. G. Clarke and Louis R. M. Del Guercio. New York, Toronto, London, The Blakiston Division, McGraw-Hill Book Company, Inc., 1956. ix, 245 pp. $6.50.

This excellent little volume proclaims itself to be "a short text and reference on the essentials of urology, presenting this science as an area within the study of general biology and medicine, rather than as a specialty." Consisting of 18 chapters, this work is divided into three main areas, The Diagnosis of Genito-Urinary Disorders, Physiologic Disturbances, and Principles of Treatment. Amply illustrated, and with a fine collection of x-rays accompanied by the patients' histories, it reads easily. The bibliography is pertinent and up to date.

This book is highly recommended to students who have need of a brief, definitive text during their short tenure on the urology wards, and to more advanced workers in the field who might profit by a proper view of this specialty as but a small segment of medicine as a whole.

J. C. C.

1956 Medical Progress. A Review of Medical Advances during 1955. Morris Fishbein, Ed. New York, McGraw-Hill Book Co., Inc., 1956. 389 pp.

In the preface it is said that this volume "is designed to review the progress made in medicine during the preceding year." The book consists of an introductory section written by the editor and twenty sections by contributors. These sections correspond roughly to the various medical and surgical subspecialties. About one half of the book is devoted to medical subjects, slightly less than one third to surgery, and the remainder to psychiatry, laboratory, and physical medicine and rehabilitation. With one exception, the sections this reviewer is particularly qualified to evaluate are

accurate, and probably as readable as this type of book can be. Adequate bibliographies are provided, except as indicated below. The book is not large, and considering the immensity of the subject it would be unrealistic to expect a very comprehensive analysis. One contributor observed that "we are forced to range widely and pause but briefly at any one particular point."

The editor, in a glowing introductory chapter, indicates incorrectly that sulfadiazine is as effective as penicillin in the treatment of streptococcal infections to prevent rheumatic fever. It is to be hoped that readers of this section will also read the section on rheumatic diseases where they will find the correct information. No other errors as important as this were detected. This introductory chapter is a condensation of the book itself, and the facts are so oversimplified that it adds nothing of value.

In the section on laboratory procedures ten rapidly advancing fields of clinical chemistry and immunology are discussed. A large amount of space is used in detailed descriptions of procedures. This space might better have been used for additional discussion of principles since the volume will not serve as a reference on laboratory methods. The bibliography provided in this section is inadequate. The sections on new drugs and neuropsychiatry provide no references at all.

The chief value of this book to a practitioner would presumably be in bridging the gap between the standard reference texts and the present time. The information provided would often be lacking in sufficient detail to be useful in a specific situation. The references provided are especially important for this reason, and uniform style in listing the titles would facilitate their use. Perhaps the most important feature of this volume is that it includes a bibliography of more than twelve hundred selected titles.

GEORGE J. ERIOU

ADDITIONAL BOOKS RECEIVED

General Biology. By Harrington Wells and Patrick H. Wells. New York, McGraw-Hill Book Company, Inc., 1956. vii, 520 pp. $6.50.

A Textbook of General Physiology. By Philip H. Mitchell. New York, McGraw-Hill Book Company, Inc., 1956. vii, 885 pp. $10.50.

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Connective Tissues. Transactions of the Fifth Conference, February 8, 9, and 10, 1954. Charles Ragan, Ed. New Jersey, Josiah Macy, Jr. Foundation, 1954. 222 pp. $4.25.

Tissue Culture Technique in Pharmacology. Roy Waldo Miner, Ed. Annals of the New York Academy of Sciences. Vol. 58, Art. 7, Pages 971-1326. New York, 1955. $4.50.

Perspectives and Horizons in Microbiology. Selman A. Waksman, Ed. New Brunswick, N. J., Rutgers University Press, 1955. x, 220 pp. $3.50.

The Chemistry of Lipids of Biochemical Significance. By J. A. Lovern. New York, John Wiley & Sons, 1955. xiii, 132 pp. $1.75.

The Truth About Cancer. By Charles S. Cameron. New Jersey, Prentice-Hall, Inc., 1956. xix, 268 pp. $4.95.

The Technique and Significance of Oestrogen Determinations. P. Eckstein and S. Zuckerman, Eds. New York, Cambridge University Press, 1955. 96 pp. 48s.

New Lives for Old. By Margaret Mead. New York, William Morrow & Co., Inc., 1956. xxi, 548 pp. $6.75.

Cells and Societies. By J. T. Bonner. New Jersey, Princeton University Press, 1955. 234 pp. $4.50.

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THE INDEX

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